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VOL. 54 JULY, 1959 NO. 1

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Clinical and Experimental

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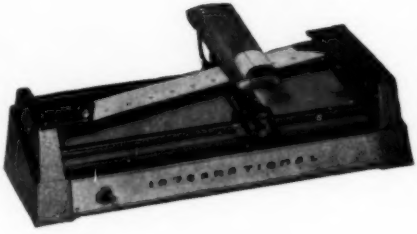

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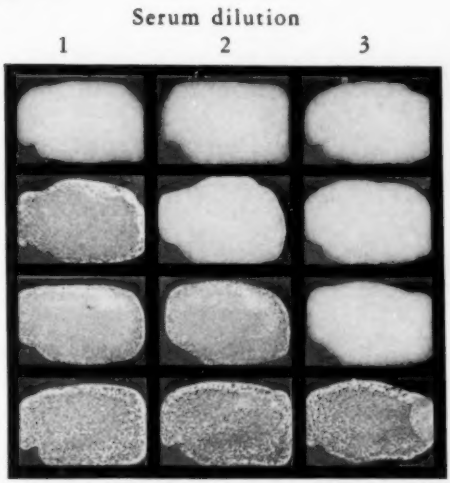

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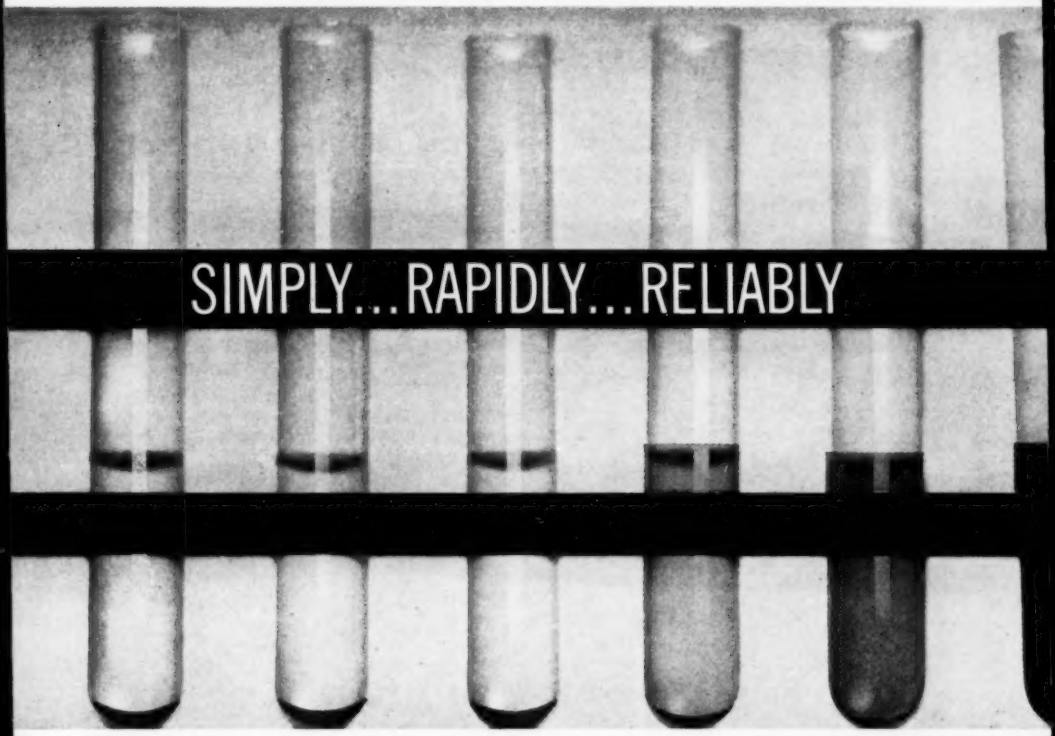

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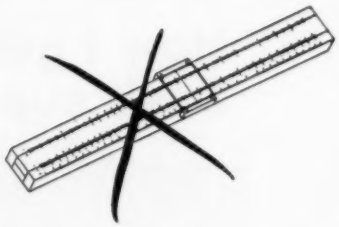

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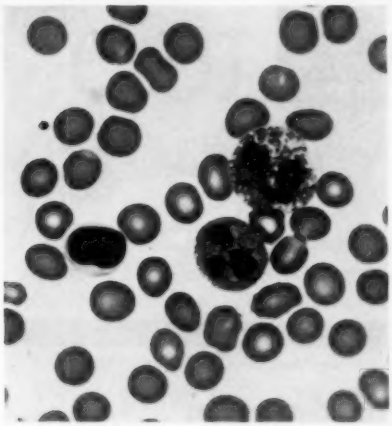
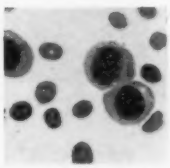
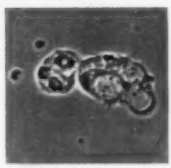


Among the red cells, three of the principal types of white cells, left to right: a lymphocyte, representing about 30% of the white cells; a neutrophil, representing 60%; an eosinophil, representing about 2%.



Megaloblastic cells, characteristic of the pattern of red cell maturation in pernicious anemia.



Erythrophagocyte, seen in an uncommon blood condition, where white cells swallow red cells. Here is one that has eaten three.

The objective documentation of hematological observations through properly controlled photomicrography is treated with some depth in the current issue of our periodical *Medical Radiography and Photography*. It contains some excellent color photomicrographs, including a series which depicts the maturation of human blood cells. However, if we were to go to the expense of reproducing the color on this page, it would use up money better spent in face-to-face instruction in hematological photomicrography for those who need it. You can look at the color all you want to by requesting a copy of the blood issue of *M.R. & P.* from Eastman Kodak Company, Medical Sales Division, Rochester 4, N. Y.

Chelatometry rolling

Though these lines were written on a chilly evening in April, they are very likely being read when it's too hot for tedious ruminations on chelatometry. All we want to do is hop on the bandwagon. Between the writing and the reading, Hahnemann Medical College & Hospital honored Chelation on May 6, 7, and 8 by a capital "C" and a symposium on "Metal-Binding in Medicine." When these things pick up momentum, none dare stand in the way.

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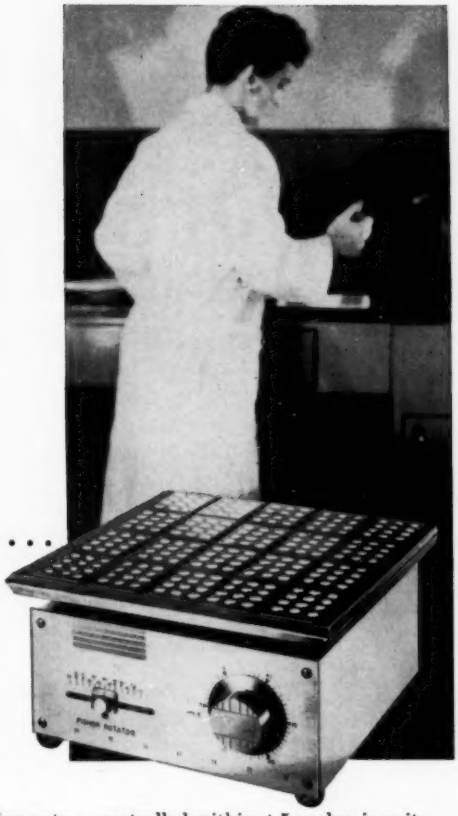

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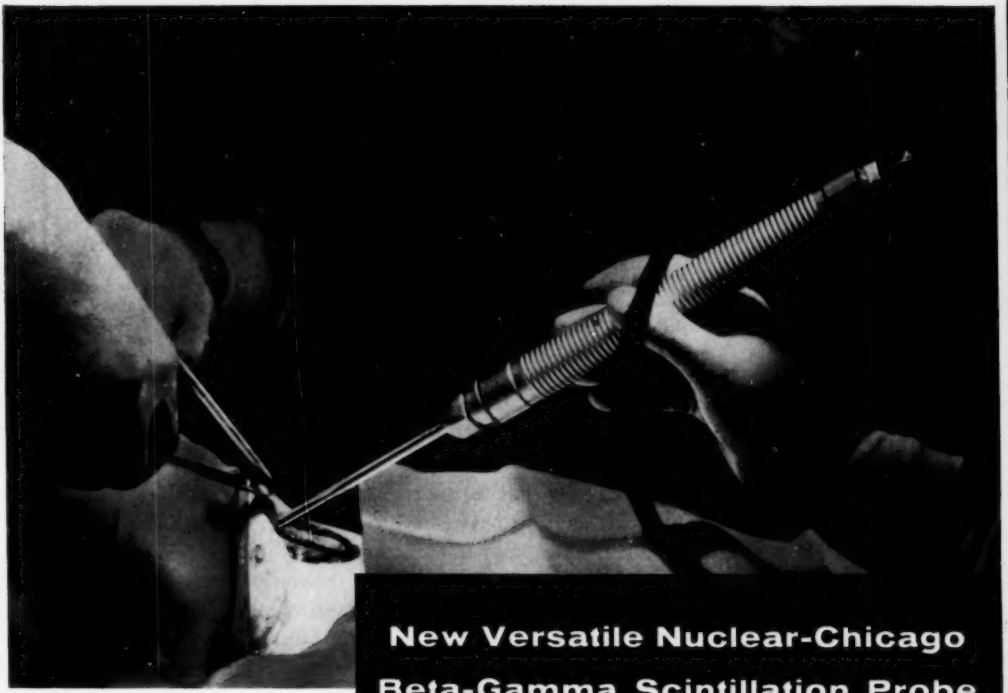

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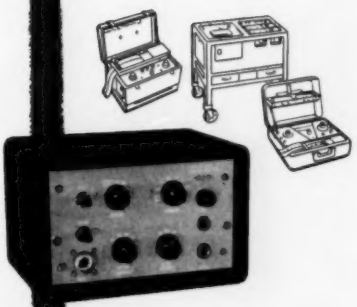

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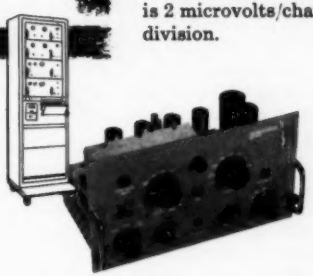

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CLINICAL AND EXPERIMENTAL

COMPOSITION OF THE UROBILIN GROUP IN URINE, BILE, AND FECES AND THE SIGNIFICANCE OF VARIATIONS IN HEALTH AND DISEASE

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MINNEAPOLIS, MINN.

WITH THE TECHNICAL ASSISTANCE OF MARY WEIMER, B.S.

THE history of the urobilin problem has been considered in some detail in recent papers[1] and need not be repeated at present except for a brief discussion of certain confusing aspects of terminology.

Jaffé[2] first used the term urobilin to describe a substance, found in the urine and bile, characterized by a well-defined absorption band and by green fluorescence with zinc. Although Van Lair and Masius[3] applied the term stercobilin to a closely similar compound obtained from human feces, it appears that MacMunn[4] first reported spectroscopic evidence for the occurrence of at least two differing substances of this type. As will be fully evident in the following it is quite impossible now to determine which of the urobilins or what combination any of these investigators were studying.

Fischer's[5] crystallization of mesobilirubinogen in 1911 marked the first outstanding advance in knowledge of these substances. This compound is the colorless, Ehrlich's aldehyde-reacting chromogen obtained by the sodium amalgam reduction of bilirubin. According to Lemberg and Legge's[6] system of nomenclature mesobilirubinogen is a mesobilane, stercobilinogen, a tetrahydromesobilane. The relationships of these compounds and other similar bilirubinoids will be considered later. Mesobilirubinogen was shown by Fischer

From the Department of Medicine, University of Minnesota Hospital, Minneapolis, Minn. Aided under a contract with the Office of the Surgeon General, United States Army. Received for publication Aug. 7, 1958.

and Meyer-Betz[7] to be identical with a crystalline urobilinogen isolated from urine of a patient with cirrhosis of the liver. This important demonstration has been chiefly responsible for the restricted use of the term urobilinogen, as synonymous only with mesobilirubinogen. Historically, however, the terms urobilin and urobilinogen relate to a group of pigments having similar absorption spectrum and green fluorescence whose chromogens exhibit the Ehrlich aldehyde reaction. Had Fischer and Meyer-Betz[7] worked with a urine sample in which stercobilinogen was preponderant, rather than mesobilirubinogen, they would have been forced to the conclusion that urobilinogen and mesobilirubinogen differ, or, if the terms were to be used as strictly synonymous, they would have been obliged to designate the Ehrlich reacting chromogen from the urine in some empirical manner, as at that time information on other members of the urobilinogen group was unavailable. In retrospect it seems highly likely that at least some and quite possibly the majority of Jaffé's urobilin was what we now know as the levorotatory stercobilin, henceforth designated as l-S; conversely, much of what Van Lair and Masius designated stercobilin may have been i- or d- forms, depending on individual circumstances, as discussed later. The group designation is believed essential from the clinical chemical standpoint, there being at least 3 chemically distinct members of the group, occurring in varying proportion in natural material. As will be apparent in the following, the intragroup variations do not have added clinical or physiologic significance when the total amount has been determined; thus, it is unlikely that any clinical chemical objectives will require separate determination of the individual members.

The isolation of crystalline stercobilin from feces and urine[8-14] provided a starting point for delineation of the other members of the urobilin group. Stercobilin was shown to differ from the urobilin immediately derived by dehydrogenation of mesobilirubinogen in vitro. The latter was first designated as "k-urobilin"[12-14] later urobilin IX,α.[15] It should be emphasized that the IX,α designation is not well suited to distinguish members of the urobilin group, as all bilirubinoids derived from protoporphyrin IX by loss of its α methene bridge have the same configuration.[51] Unlike mesobilirubinogen or its urobilin (i-U), stercobilin was found not to yield methyl ethyl maleinimid on vigorous oxidation.[9] Stercobilin is strongly levorotatory while i-urobilin, as obtained from mesobilirubinogen, is optically inactive.[17] Stercobilinogen, although easily formed by reduction of stercobilin in vitro, could not be crystallized while mesobilirubinogen (i-urobilinogen) crystallizes with ease under the same conditions.[9] The first instance in which crystalline stercobilin and mesobilirubinogen were isolated from the same urine sample, was in a case of hemolytic jaundice.[14] The two substances were present in approximately equal amounts.

In recent papers,[1] mainly on behalf of simplicity in discussing the clinical chemistry and terminology of the urobilin group, the 3 members have been designated d-, i-, and l- on the basis of their widely differing optical activity, but fully recognizing that they are not isomers. This is open to the objection[16]

that d- and l- are not isomers, nor i- a racemer. Siedel prefers to restrict use of the term urobilin to the inactive form, retaining the suffix IX,α (see above), and designating the l- form only as stercobilin, the d- form as dehydrourobilin d.* The author[1c] has recently used a similar designation, i.e., d-D-Urobilin to express both the dextrorotatory activity and the dehydro character of the d- as compared with the i- form. However, this is cumbersome and might imply that d- is derived from i- by dehydrogenation. As will be seen, the available evidence indicates that this is quite unlikely. It is clear that any final nomenclature must await full knowledge of the structure of the 3 compounds. In the meanwhile it is believed preferable to define them simply on the basis of optical activity and number of hydrogen atoms in the molecule: d-urobilin (H_{40}), i-urobilin (H_{42}), and l-stercobilin (H_{46}). The respective chromogens or urobilinogens each have two additional hydrogens. The abbreviations d-U, i-U, and l-S, or d-Ubg, i-Ubg, and l-Sbg will be used in this sense throughout.

d-U was first encountered in samples of infected fistula bile.[18] Here it appeared that an ordinary coliform or fecal type of flora reduced bilirubin to the d- rather than the i- or l- types. More recently d-U has often been observed in the feces of patients receiving or who have recently received broad spectrum antibiotics such as tetracycline or erythromycin.[19-21] It has also been encountered in the excreta of certain patients who had not, so far as could be determined, received any antibiotics.[1a] Gray[22] recently reported its isolation from the feces of a case of thalassemia in which no history of antibiotic administration was obtained.

The enterogenous concept of urobilin formation was based on the studies of Maly,[23] von Müller,[24] and the later, extensive experiments of McMaster and Elman.[25-27] In recent years some confusion has been introduced in the urobilin literature, because of a dichotomous concept of origin advanced by Baumgärtel and others.[28-35] According to this, mesobilirubinogen (the chromogen of i-U) is formed only in the liver or biliary tract, more particularly in the presence of bile stasis in the damaged liver, and stercobilinogen is formed only by the action of the bacterial flora in the colon. This theory would specifically interdict a conversion of the one to the other, although evidence for this transition had been described in an earlier paper[36] long antedating the Baumgärtel hypothesis. The conversion of N_{15} mesobilirubinogen to stercobilinogen by fecal bacteria in vitro has been reported more recently by Lowry and associates.[37] To explain the well-known absence of urobilinogen in the urine in cases of complete biliary obstruction, Baumgärtel postulated an "enzyme block," or suppression of the hepatic dehydrogenase presumed essential for the conversion of bilirubin to mesobilirubinogen in the liver or bile. This "block" was supposed to be affected by a sufficient elevation of the serum bilirubin. Thus it appears that Baumgärtel's concept requires a certain degree of bile stasis, but not too much. It did not take into

*Gray[60] has recently described an inactive isomer of d-urobilin, also a dextroisomer of i-urobilin; this emphasizes the need of an exact terminology.

adequate account the careful studies of McMaster and Elman.[25-27] In these it was shown clearly that urobilin disappeared from the urine and feces of dogs if bile was prevented from entering the intestinal tract, either by complete obstruction or total external fistula. Liver injury under these situations failed to produce urobilinuria, although it was regularly observed in the intact animals with the same form of injury and similar degrees of hyperbilirubinemia. Billi and associates[38] found no urobilin formation after exenteration in dogs except as a late sequel to establishment of a fecal flora in the stomach.

Baumgärtel, Stich,[31, 32] Rudolph,[33] and Maier[34] have published data believed to show that in the presence of liver injury mesobilirubinogen or its corresponding i-U is preponderant in the urine, and that in normal individuals and patients with hemolytic or pernicious anemia, the l- form, stercobilin, is usually preponderant. Brandt[39] recently reported somewhat similar findings, but concluded that both mesobilirubinogen and stercobilinogen are enterogenous. In all of these studies the d- form was disregarded, the method of distinguishing urobilin and stercobilin, in other words, the i- and l- forms, depending upon mesobiliviolin formation either by $FeCl_3$ or the pentdyopent reaction, both characteristic of i- but not of l-. The d- form is not distinguished from i- by either reaction as hitherto employed. Furthermore, when the reaction is used qualitatively as it was by these investigators, the presence of even a minor fraction of i- or d- is misleading because of the relative intensity of the violet or blue color which masks the color of the unchanged stercobilin. The $FeCl_3$ reaction is considered more fully in the following; the pentdyopent reaction has also been studied in detail but has not been used in the present study as, in our hands, it has been inconsistent and unreliable even for qualitative purposes.

Gohr and associates[35] reported that urobilinuria persisted in patients with liver disease who were receiving Aureomycin, but they apparently did not consider that this might be d-U, which, as noted later, may be present indefinitely in the urine of patients receiving broad spectrum antibiotics.

The principal objectives of the present study have been (1) development of an improved method for approximate quantitative distinction of the 3 members of the urobilin group, and (2) application of this method to various samples of urine, bile, and feces, both in health and disease, with special reference to liver injury and hemolytic states.

MATERIAL AND METHODS

The representatives of the urobilin group in any given sample of feces were extracted, concentrated, at least partially purified and often crystallized by the alcohol-Al_2O_3-water-petroleum ether method previously described.[40] Urine and bile samples were adjusted to pH 4.0 to 5.0 (acetic) and then extracted directly with petroleum ether (B.P. 30° to 60° C.), the bile samples being extracted after preliminary fivefold dilution with distilled water. Emulsions were broken by addition of a small amount of 95 per cent ethanol, followed by a single quick shake of the separatory funnel. As noted in the flow diagram in Fig. 1 the collected petroleum ether extract was washed once with distilled

water then shaken repeatedly with dilute iodine solution to convert any urobilinogen to its respective urobilin, which at once enters the aqueous phase while the iodine is extracted by the petroleum ether. This process is repeated until the water is nearly or quite colorless. The pooled aqueous fraction is washed twice with peroxide-free ethyl ether, then acidified with 7.5 N HCl to yield a final concentration of about 1.5 N. The urobilin group is removed from the hydrochloric acid by successive chloroform extraction. The $CHCl_3$ solution is dried by filtration, concentrated to a convenient volume under reduced pressure, and mixed with 10 volumes of petroleum ether. The precipitated urobilin group is collected on a small filter paper and redissolved in a few milliliters of anhydrous chloroform. The concentration is then determined in an Evelyn photoelectric colorimeter using a 490 filter and a standard optical density curve prepared with crystalline stercobilin hydrochloride. Crystallization from chloroformacetone or methyl alcohol-ethyl acetate[41] is usually successful if the amount exceeds 200 μg.

Optical activity measurements were made in a majority of instances. A Rudolph polarimeter sensitive to 0.001 degree was used. The readings were made with a specially constructed photoelectric device to eliminate the visual comparison.* The accuracy of the optical activity measurement was carefully analyzed† with repeatedly crystallized i-urobilin hydrochloride prepared by the sodium amalgam reduction of bilirubin and the I_2H_2O-petroleum ether method.[41] With decreasing concentration an increasing error on the levo-side was observed, as seen in Table I.†

TABLE I

MG. I-UROBILIN /100 ML. $CHCl_3$	$[\alpha]_D^{20}$ MEAN DEGREES	± 2 S. E. OF MEAN, IN DEGREES	± 3 S. E. OF MEAN, IN DEGREES	ACTUAL MEASURED ROTATION (AVERAGE)
25.5	0	−23 to +23	−35 to +35	0.000
	−8	−31 to +15	−43 to +27	−0.002
15.7	−31	−66 to +4	−84 to +22	−0.005
	−38	−65 to −11	−79 to +3	−0.006
9.1	−87	−132 to −42	−155 to −19	−0.008
	−109	−141 to −77	−157 to −61	−0.010
4.2	−144	−218 to −70	−255 to −33	−0.006
	−166	−347 to +15	−437 to +105	−0.007
2.4	−700	−854 to −546	−931 to −469	−0.017
	−740	−991 to −489	−1116 to −364	−0.018

The data shown in Table II were compared with the analysis of significance, the results of which are given in Table I. Values indicated with an asterisk are regarded as insignificant or doubtful (± 3 S. E. of the mean).

While in general the optical activity measurements agree reasonably well with the results of $FeCl_3$ oxidation, certain unexplained discrepancies have been encountered and will be considered later.

After determining the concentration of the final chloroform solution and measuring its optical activity, $FeCl_3$ oxidation was carried out according to a modification of the methods of Fischer and Niemann[42] and Legge.[43] The former was the first method described for the preparation of mesobiliviolin from crystalline mesobilirubinogen. Legge's

*Designed and constructed by R. T. Bruss. The apparatus employs a photometer with a selected IP 21 electron multiplier phototube. An accelerating potential of 880 volts is supplied by a regulated power supply. The output of the phototube is connected to an electrometer amplifier with full scale sensitivity of 0.035 microamperes. The sum of the phototube dark current and amplifier instability is less than 1 per cent of this value. The photometer is sufficiently sensitive and stable to permit measurements of 0.001 degree with samples having 2 per cent or more light transmission. A sodium vapor light source with regulated power supply is used. Readings are taken as the mean of two angles on opposite sides of the null point, of equal light intensity. Sources of instability are the sample holding tube, especially with chloroform solutions, also the slow thermal drift of the sodium lamp. The former is avoided with tubes having one end enlarged for air bubbles and central receptor. The latter is minimized by maintaining the room temperature at 70 to 72° F.

†Dr. Ivan Frantz kindly assisted with this analysis.

method was intended primarily to distinguish urobilin IX,α (i-U) and stercobilin. It did not take the d- compound into account, nor any of the oxidation products of i- and d-, except mesobiliviolin (to be discussed later).

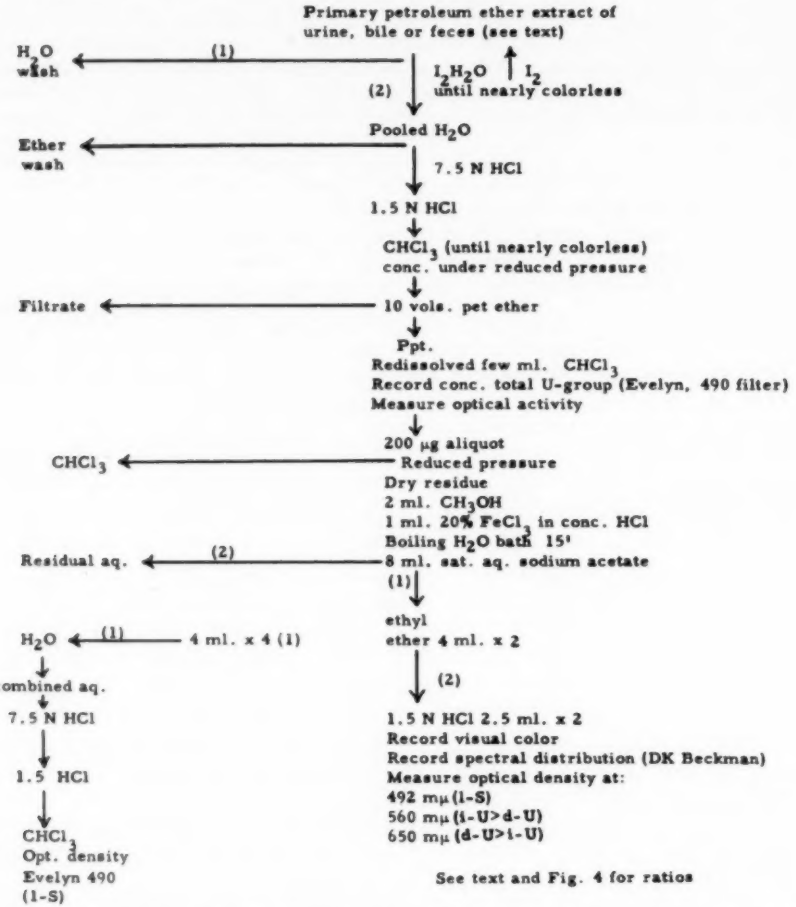

Fig. 1.—Flow diagram of $FeCl_3$ oxidation method for semiquantitative distinction of d-, i-, and l- members of naturally occurring urobilin group.

A sufficient volume of the chloroform solution is used to provide 200 µg of urobilin group, based on absorption at 490 mµ as mentioned previously. The method employed is shown in the flow diagram in Fig. 1. The chloroform solution is evaporated to dryness on the water bath and the urobilin group redissolved in 2 ml. of methanol to which is added 1 ml. of 20 per cent $FeCl_3$ in concentrated HCl. This solution is kept in a boiling water bath for 15 minutes then transferred to a separatory funnel. Eight milliliters of saturated aqueous sodium acetate solution is added, and the mixture is at once shaken with 2 successive 4 ml. portions of ethyl ether. The aqueous fraction is saved, the combined ether washed 4 times with small amounts of water, all of which is added to the aqueous fraction. The ether is next extracted twice with 2.5 ml. amounts of 1.5 N HCl.

The color of this solution is noted, its volume made up to 20 ml., and its spectral distribution then recorded with a DK Beckman self-recording spectrophotometer, in the range of 375 to 900 mμ. The curves thus obtained are compared with those derived by similar treatment of the 3 pure compounds as well as a number of standard mixtures, as seen in Fig. 2. The per cent absorption at 492, 560, and 650 mμ were recorded to gain information as to the relative amounts of unchanged pigment, mainly l-stercobilin, and of mesobiliviolin-mesobilirhodin (henceforth designated as MvMr) and glaukobilin (Gl), respectively. These compounds were prepared from mesobilirubinogen by Siedel and Möller's[44] method in order to permit study of their spectral distribution and optical densities. As noted in Fig. 3, there is considerable overlap in the absorption of MvMr and Gl. The absorption at 560 to 580 mμ is related to a variable mixture of Mv and Mr.

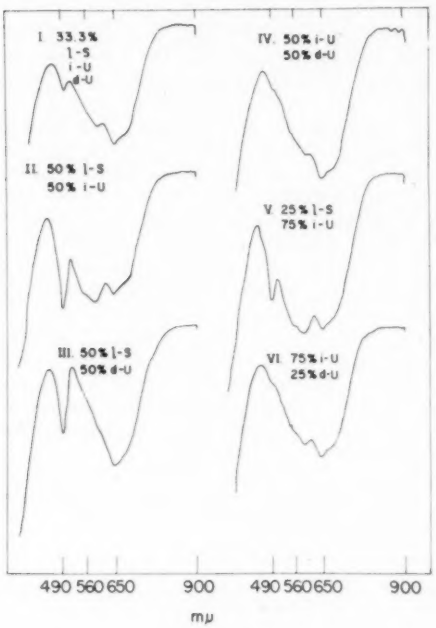

Fig. 2.

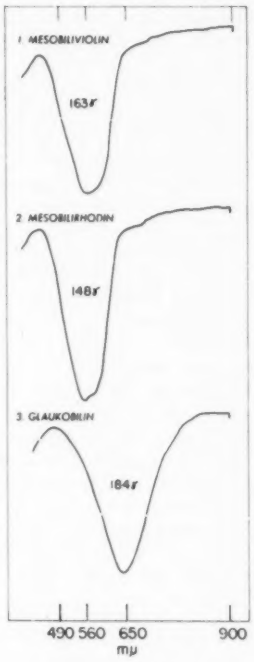

Fig. 3.

Fig. 2.—Spectral distribution curves of products of $FeCl_3$ oxidation of the 3 members of the urobilin group and various mixtures. Beckman DK spectrophotometer, drive 10; optical density increasing from above downward. A total of 200 μg was used in each of the runs shown, with the proportions as indicated.

Fig. 3.—Spectral distribution curves of mesobiliviolin, mesobilirhodin, and glaukobilin. The amount shown was dissolved in each instance in 20 ml. 1.5 N HCl. Beckman DK spectrophotometer, drive 10; optical density increasing from above downward.

As seen in Fig. 3 these compounds have similar, but not identical, absorption. Thus, the values recorded are of relative rather than absolute significance. It is also evident from the curves shown in Fig. 3 that significant absorption at 492 mμ is produced by MvMr when present in relatively high concentration. This represents only the outer margin of a diffuse absorption band, not a separate band with maximum at or near 492, such as characterizes unchanged l-S. It should be emphasized that l-S is not converted to MvMr or Gl, and remains in the aqueous phase, hence relatively little is present in the final HCl. Nevertheless, if l-S was significantly represented in the original 200 μg of urobilin group

subjected to the oxidation, enough was always present in the final HCl to provide a definite peak or at least a bulge in the spectral distribution curve at maximum 492 mμ. It should be noted that in the $FeCl_3$ reaction a considerable part of the stercobilin completely disappears and is unaccounted for.

It is seen in Fig. 2 that the $FeCl_3$ oxidation of i-U yields a variable mixture of MvMr and Gl, the former invariably present in considerable amount. Oxidation of pure d-U under the above conditions yields mainly glaukobilin; as a rule the spectral distribution curve (Fig. 2) is that of pure Gl and little or no MvMr type absorption is seen. It should be noted, however, that both d- and i- are converted primarily (1.5, 3 minutes' heating) to MvMr but for reasons not yet clear, transition to glaukobilin is then relatively retarded with i-, but not with the d- form. Glaukobilin ester was prepared individually from mesobilirubinogen, i-U and d-U, and crystallized from chloroform-methanol. (M. P. 224° C., no depression on mixture.) Glaukobilin had not previously been prepared from the d-compound. Glaukobilin hydrochloride has a greenish-blue color and has been termed mesobiliverdin by Lemberg.[6] This corresponds with the aqua color of the final HCl solution after $FeCl_3$ oxidation of d-U, as above described (Fig. 1). i-U under the same conditions yields a color varying from purple to royal blue depending on the proportion of MvMr and Gl. This will be apparent in the results given later. The color of the final HCl solution obtained after $FeCl_3$ oxidation of l-S (stercobilin) is usually a weak pink or faint salmon. These color differences are characteristic of the final solution when 200 μg of starting material is heated with $FeCl_3$ for 15 minutes, as was customarily done (Table II). With smaller amounts the color differences may not be as outspoken.

A minute amount of MvMr type absorption is commonly noted at maximum 560 to 580 mμ, even with the purest samples of l-S, repeatedly recrystallized from chloroform acetone, then from methyl alcohol-ethyl acetate.[41] This is probably due to the presence of small amounts of i-U in the l- material, quite possibly bound as a molecular complex. Further support for this view has recently been gained in the observation that following reduction of such samples of l-S by fecal bacteria, in broth cultures, the crystalline material no longer yields even traces of MvMr, on $FeCl_3$ oxidation. This agrees with the finding that under appropriate conditions pure i-U is reduced in part or completely to l-S by fecal bacteria.[1c] In earlier communications[10, 45] it was stated that stercobilin yields mesobiliviolin under suitable conditions of oxidation. Our present results indicate that this was due to admixture or complexing of small amounts of i-U in the sample.

Following $FeCl_3$ oxidation of carefully purified samples of the 3 forms, and various standard mixtures, the following ratios of absorption were calculated and plotted as shown in Fig. 4: $\frac{492\ (l\text{-}S)}{560\ (MvMr) + 650\ (Gl)}$, $\frac{560\ (MvMr)}{650\ (Gl)}$.

The first of these ratios provides reasonably decisive information as to the proportion of l- versus i- + d- forms in the original mixture. It will be evident in the results given later that wherever this ratio has indicated a large proportion of l-S, according to the plot in Fig. 4, there has also been a proportionately large amount of residual absorption at 492 mμ in the pooled aqueous fraction (Fig. 1 and Table II, column 27). The second ratio is less decisive, due to greater overlap of absorption (Fig. 3), but it is evident that it provides satisfactory data as to proportion of i- and d- forms in the original mixture. It is seen that with i- alone or i- + l- the ratio is 0.85 or greater, while with d- alone or d- + l- the ratio is less than 0.6. The ratios correspond well with the patterns of absorption shown in Fig. 2.

The above methods were applied to the study of 104 samples from 68 individuals. The samples included 40 (24 hr.) urine collections from 38 individuals; 26 samples of feces, mainly 4-day collections; 8 bile samples from 5 cases; 2 samples of duodenal drainage bile from 2 cases; contents of the cecum from 12 cases and from the sigmoid in 10 of the same individuals.*

*Grateful acknowledgment is made to Dr. William Guthrie, Coroner of Hennepin County, Minneapolis, Minn., for the provision of this material and pertinent information.

Bile samples from Cases 66 and 68 (Table II) were sterile; that from Case 65 contained *Pseudomonas acruginosa*. The bile used in Case 62 was not cultured but it was obviously overgrown with a fecal flora as judged by turbidity and odor. The bile in Case 64 was clear and ordorless. In 7 of the 9 cases from which contents of the cecum and sigmoid were examined, there was no history of antibiotic intake or administration. All of these were instances of sudden death without previous recent illness or medical treatment.

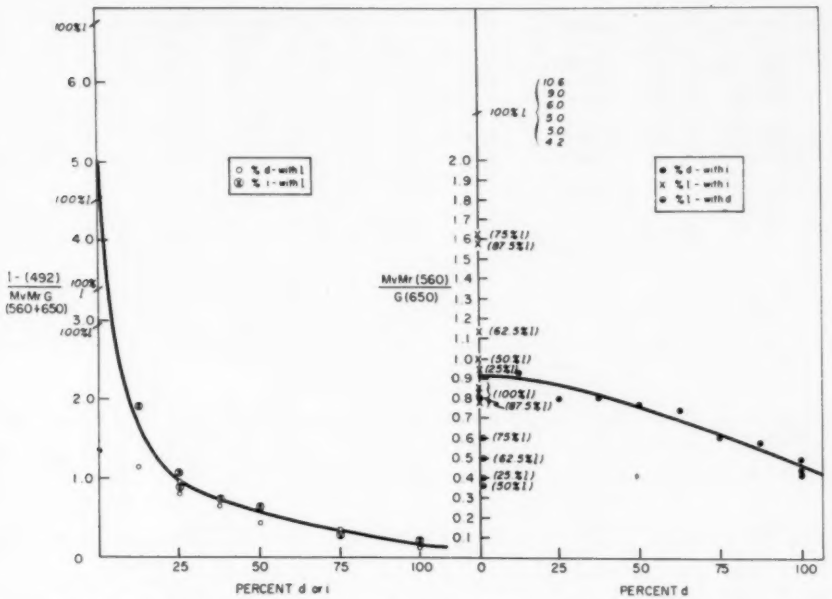

Fig. 4.—Plot of ratios obtained with various mixtures of d-, i-, and l- members of the urobilin group.

$$(1)\ \frac{\text{abs. } 492\ m\mu\ (l\text{-}S)}{\text{abs. } 560\ m\mu + 650\ m\mu\ (i\text{-}U + d\text{-}U)} \quad \text{and} \quad (2)\ \frac{\text{abs. } 560\ m\mu\ (i\text{-}U > d\text{-}U)}{\text{abs. } 650\ m\mu\ (d\text{-}U > i\text{-}U)}$$

RESULTS

Table II includes the data on the composition of the urobilin group in urine, feces, and bile, both in normal individuals and in patients with diseases, especially hepatic and hemolytic disorders. For reasons mentioned before, the latter were relatively emphasized in view of the previous reports that these two groups of conditions differ in respect to a preponderant type of urinary urobilin. It is clearly evident from the present data that there is no consistency in this regard.

In general, within rough limits, the results of optical activity measurement and the $FeCl_3$ oxidation method are in agreement. Of 59 samples in which optical activity was measured, there was disagreement in 8 (samples 11b, 14b, 30a, 47a, 48a, 49a, 55a, and 62a). In 4 of these (Nos. 14b, 30a, 47a, and 48a) the readings were significant (see Table I) and in the direction corresponding with the results of $FeCl_3$ oxidation, but the values were too small,

TABLE

CASE NO.	PATIENT, SEX, AND AGE	DIAGNOSIS	ANTIBIOTIC* AND DATE LAST TAKEN	MATERIAL EXAMINED				POLARIMETRY	
				SAMPLE	DATE	SOURCE	CRYST. OR TOTAL	Mg. % IN $CHCl_3$	NET READING IN DEGREES
1	D. L. M 23	Normal	None since 4/56	a	1/16/57	Urine	T		
2	J. M. M 32	Normal	None	a	1/17/57	Urine	T		
3	R. R. M 24	Normal	None	a	1/21/57	Urine	T		
				b	4/ 5/57	Urine	T		
4	M. R. F 21	Normal	None	a	1/21/57	Urine	T		
				b	4/ 5/57	Urine	T		
5	J. L. M 24	Normal	None	a	1/29/57	Urine	T		
6	D. H. M 24	Normal	None	a	3/17/57	Urine	T		
7	S. G. F 29	Normal	1 year ago	a	1/18/57	Feces	C	4.15	-0.153
				b	10/26/56	Feces	C		
8	A. L. F 62	Normal	None known	a	2/15/57	Feces	T		
9	O. S. M 56	Hodgkin's disease, hemolytic anemia	1953, elsewhere	a	9/24/54	Feces	C	4.2	-0.144
10	F. M. M 49	Cirrhosis; Ca esophagus	None	a	8/ 5/54	Urine	C		
				b	8/ 5/54	Feces	C	9.6	-0.395
11	L. S. F 63	Cirrhosis	P 8/7/54	a	8/ 6/54	Urine	C	4.1	+0.061
				b	8/ 9/54	Feces	C	1.8	-0.013
12	M. F. F 64	Pernicious anemia, heart failure	None	a	9/24/54	Urine	C	4.4	-0.188
13	C. G. M 47	Cirrhosis	P 11/30/54	a	11/30/54	Urine	C	3.55	-0.068
14	A. C. M 49	Cirrhosis	None	a	12/17/55	Urine	C		
				b	12/12/55	Feces	C	3.75	+0.011
15	J. R. M 74	Subtotal gastrectomy	PT 4/3/54	a	4/14/54	Urine	C	4.4	+0.110
16	H. J. F 72	Endometrial hyperplasia	None since 1950	a	11/10/55	Urine	T	2.8	-0.076
				b	11/10/55	Feces	T		
17	A. C. M 49	Carcinoma pancreas	None	a	11/17/55	Urine	T	1.25	+0.076
18	L. H. F 58	Chronic hepatitis	None	a	11/ 3/55	Urine	C		
19	E. B. F 21	Postnecrotic cirrhosis	None	a	5/ 1/56	Urine	T	4.08	
20	W. K. M 58	Laennec's cirrhosis	None	a	6/18/56	Urine	T	4.08	-0.076
21	A. J. M 75	Carcinoma head of pancreas	None	a	9/17/55	Urine	T	2.58	-0.060
				b	9/28/55	Feces	T	5.12	-0.174
22	G. H. M 71	Cholelithiasis Cholecystectomy	P, S 7/30/56	a	8/ 5/56	Urine	T	4.05	-0.147
23	H. C. F 81	Carcinoma ampulla Obst. jaundice	None	a	12/10/56	Feces	T	3.8	-0.107
24	A. B. F 38	Refractory anemia	None	a	12/31/56	Feces	T	2.2	-0.083
25	W. N. M 58	Myocardial infarct	P 11/11/54 S 11/21/54	a	11/23/54	Urine	C	4.5	-0.165
26	J. S. F 6	Hemolytic anemia	None	a	12/ 6/55	Urine	T	2.31	+0.076
				b	12/ 3/55	Feces	T	2.4	-0.025
				c	12/ 3/55	Feces	C	4.4	-0.064
27	G. S. M 5	Hemolytic anemia	None	a	12/ 3/55	Feces	T	3.2	-0.028
				b	12/ 3/55	Feces	C	5.0	-0.088

$[\alpha]_D^{20}$	CASE AND SAMPLE NO.	ABSORPTION: μg TOTAL U GROUP OXID. $FeCl_3$	PER CENT ABSORPTION mμ: 492	560	650	ABSORPTION: 492 / 560 + 650	ABSORPTION: 560 / 650	APPROXIMATE PER CENT OF d, i, l FORMS FROM ABSORPTION RATIOS (FIG. 4): d	i	l	COLOR OF FINAL SOLUTION	μg U IN RESID. AQUEOUS
	1a	71	10	18	31	0.2	0.58	90	10		Pale blue	2
	2a	43	16	8	4	1.3	2.0		15	85	Pale pink	15
	3a	200	52	85	57	0.36	1.49		65	35	Purple	19
	3b	200	37	52	18	0.53	2.89		50	50	Purple	41
	4a	18	11	6	4	1.1	1.5		20	80	Colorless	7
	4b	102	30	12	5	1.76	2.4		10	90	Pink	30
	5a	200	40	36	12	0.83	3.0		30	70	Pale purple	49
	6a	29	12	15	12	0.44	1.3		60	40	Colorless	1
3,686	7a	200	47	43	10	0.89	4.3		25	75	Pink-purple	29
	7b	200	19	47	23	0.27	2.04		75	25	Blue-purple	
	8a	200	91	60	13	1.24	4.61		15	85	Pink-purple	100
3,429	9a	200	92	14	2	5.75	7.0			100	Pink	52
	10a	200	30	43	23	0.45	1.87		60	40	Purple	
4,114	10b	200	48	19	1	2.4	19.0			100	Pink	70
1,499	11a	200	26	64	92	0.17	0.69	70	30		Blue-aqua	3
−722†	11b	200	23	56	86	0.16	0.65	75	25		Blue	3
4,273	12a	200	71	6	3	7.9	2.0			100	Pink	55
1,915	13	200	44	68	44	0.39	1.54		60	40	Purple	26
	14a	200	11	35	74	0.1	0.47	100			Aqua	2
+300	14b	200	11	37	90	0.08	0.41	100			Aqua	1
2,500	15a	200	12	47	86	0.09	0.54	90	10		Aqua	2
2,785	16a	200	49	22	2	2.1	11.0			100	Pink	40
2,960	16b	200									Pink-purple	117
4,720	17a	200									Aqua	8
3,014	18a	200	22	58	92	0.15	0.63	75	25		Aqua	4
3,259	19a	200	55	27	7	1.62	3.86		10	90	Pink	20
1,862	20a	200	22	31	18	0.45	1.74		55	45	Purple	81
2,336	21a	200	57	42	3	1.27	14.0		15	85	Purple	42
3,394	21b	200	53	10	1	4.82	10.0			100	Pink	53
3,629	22a	200	54	41	5	1.17	8.2		20	80	Purple	54
2,816	23a	200	69	18	2	3.45	9.0			100	Pink	58
-3,772	24a	200	40	53	10	0.63	5.3		40	60	Purple	50
-3,660	25a	200	87	19	2	4.14	9.5			100	Pink	
-3,290	26a	200	13	37	85	0.14	0.436	100			Aqua	2
-1,041*	26b	200	41	80	82	0.25	0.97		80	20	Blue	6
-1,454	26c	200	35	73	80	0.23	0.91		80	20	Blue	8
−875	27a	200	42	78	66	0.29	1.18		75	25	Blue	14
-1,760	27b	200	56	86	82	0.33	1.02		70	30	Blue	19

CASE NO.	PATIENT, SEX, AND AGE			DIAGNOSIS	ANTIBIOTIC* AND DATE LAST TAKEN	MATERIAL EXAMINED				POLARIMETR	
						SAMPLE	DATE	SOURCE	CRYST. OR TOTAL	Mg. % IN $CHCl_3$	NET READING IN DEGREE
28	S. S.	M	7	Hemolytic anemia	None	a	12/ 3/55	Feces	T	2.6	-0.034
						b	12/ 3/55	Feces	C	1.26	-0.033
29	D. S.	M	11	Hemolytic anemia	None	a	12/ 3/55	Feces	T	4.9	-0.162
						b	12/ 3/55	Feces	C	3.2	-0.119
						c	12/14/55	Urine	T		
30	S. W.	M	49	Serum hepatitis	None	a	7/19/53	Urine	C	4.54	-0.174
						b	7/19/53	Feces	C	5.45	-0.164
31	G. G.	M	41	Cirrhosis	P 7/53	a	9/15/54	Urine	C		
32	E. J.	F	30	Cirrhosis	7/11 to 7/15/54	a	7/29/54	Urine	C		
						b	7/29/54	Feces	C		
33	H. P.	F	70	Cirrhosis	None since 1947	a	9/26/54	Urine	C		
34	J. H.	F	19	Infect. hepatitis	10/19/54 P, S	a	10/26/54	Urine	C		
35	B. M.	F	44	Diabetes, ch. cholecystitis	N, P, S 12/17/54	a	12/18/54	Feces	C		
36	C. S.	F	71	Lymph. leukemia	1/25/54 T	a	10/10/54	Urine	C		
37	S. R.	M	50	Cirrhosis	12/7/53 P	a	12/14/53	Urine	C		
38	C. P.	F	53	Biliary cirrhosis	None	a	8/25/53	Urine	C		
39	O. H.	M	59	Undiagnosed liver disease	None	a	12/ 2/53	Urine	C		
						b	12/ 6/53	Feces	C		
40	E. N.	F	29	Infect. hepatitis	None	a	11/ 5/53	Urine	C		
						b	11/ 5/53	Feces	C		
41	L. N.	F	59	Infect. hepatitis	9/23/53 S, P	a	11/ 3/53	Urine	C		
42	E. A.	M	49	Cirrhosis, diabetes	9/6/53 S, P	a	9/ 9/54	Feces	C		
43	E. S.	M	40	Bacterial endocarditis	8/18/55 P	a	8/18/55	Feces	C		
44	M. P.	M	51	Hemolytic anemia	None	a	12/21/55	Urine	C	3.36	+0.09
						b	12/19/55	Feces	T	4.35	-0.011
45	S. D.	M	20	Infect. hepatitis	None	a	8/ 3/53	Urine	C	11.9	-0.549
46	R. Z.	M	49	Cirrhosis	None	a	12/10/53	Urine	C	4.2	-0.166
						b	12/10/53	Feces	C	3.6	-0.150
47	H. B.	M	61	Laennec's cirrhosis	7/3/53 P	a	7/ 6/53	Urine	C	4.1	-0.143
						b	7/ 6/53	Feces	C	10.0	-0.388
48	C. F.	F	19	Cirrhosis	6/16/53 P	a	6/23/53	Urine		3.15	-0.111
						b	6/20/53	Feces		5.75	-0.267
49	A. M.	F	62	Hepatic necrosis	11/18/53 P 11/24/53 T	a	12/17/53	Cecum	C	2.9	-0.019
50	G. L.	F	64	Cerebral hemorrhage	8/54, P, S, F	a	8/22/55	Cecum	T		
						b	8/22/55	Sigmoid	T		
51	E. B.	F	36	Diabetes mellitus, coronary disease	4/24/53 P	a	12/ 1/53	Cecum	T		

—CONT'D

$[\alpha]_D^{20}$	CASE AND SAMPLE NO.	ABSORPTION: μg TOTAL U GROUP OXID. $FeCl_3$	PER CENT ABSORPTION mμ 492	560	650	ABSORPTION: 492 / 560 + 650	ABSORPTION: 560 / 650	APPROXIMATE PER CENT OF d, i, l FORMS FROM ABSORPTION RATIOS (FIG. 4) d	i	l	COLOR OF FINAL SOLUTION	μg l-s IN RESID. AQUEOUS
-1,307	28a	200	49	83	40	0.39	2.1		65	35	Purple	17
-2,620*	28b	200	54	85	50	0.4	1.7		60	40	Purple	21
-3,272	29a	200	67	53	5	1.2	10.6		20	80	Pink-purple	52
3,719	29b	200	41	31	4	1.17	7.75		20	80	Pink-purple	55
	29c		34	70	75	0.23	0.93		90	10	Blue	
-3,832	30a	200	48	78	34	0.43	2.29		60	40	Blue-purple	35
-3,009	30b	200	52	19	2	2.47	9.50			100	Pink	47
	31a	200	44	41	3	1.0	13.7		20	80	Pale purple	
	32a	160	9	23	76	0.09	0.31	100			Pale aqua	
	32b	45	11	21	48	0.16	0.44	100			Pale aqua	
	33a	200	21	50	89	0.15	0.56	90	10		Aqua	
	34a	200	19	46	87	0.14	0.53	90	10		Aqua	
	35a	195	25	59	83	0.18	0.71	65	35		Blue	
	36a	200	25	62	80	0.18	0.78	50	50		Blue	
	37a	200	17	53	92	0.12	0.53	90	10		Aqua	
	38a	200	41	75	49	0.32	1.53		65	35	Purple	
	39a	200	32	66	75	0.23	0.88		90	10	Blue	7
	39b	200	45	25	3	1.61	8.2		10	90	Purple	31
	40a	100	21	50	34	0.25	1.47		80	20	Blue-purple	14
	40b	100	27	35	9	0.61	3.9		50	50	Purple	12
	41a	100	32	54	1	0.58	5.4		50	50	Purple	30
	42a	200	42	78	15	0.45	5.2		60	40	Purple	
	43a	200	9	25	63	0.10	0.39	100			Violet	
+2,678	44a	200	23	49	79	0.18	0.62	75	25		Aqua	3
-252*	44b	200	30	65	86	0.2	0.76	40	40	20	Blue	1
-4,613	45a	200	46	24	3	1.7	8.0		10	90	Pink	83
-3,970	46a	200	37	36	5	0.9	7.2		20	80	Pale purple	145
-4,175	46b	200	51	11	1	4.6	11.0			100	Pink	78
-3,487	47a	200	53	73	33	0.5	2.2		50	50	Purple	30
-3,880	47b	200	44	16	2	2.4	8.0			100	Pink	80
-3,523	48a	200	31	85	53	0.22	1.6		85	15	Purple	34
-4,644	48b	200	45	21	2	1.96	10.5		10	90	Pink	123
-655*	49a	200	15	51	92	0.10	0.55	90	10		Aqua	1
	50a	173	6	15	32	0.13	0.47	100			Aqua	20
	50b	200	62	76	33	0.57	2.3		50	50	Deep purple	37
	51a		34	11	1	2.8	11.0			100	Pink-purple	

TABLE I

CASE NO.	PATIENT, SEX, AND AGE	DIAGNOSIS	ANTIBIOTIC* AND DATE LAST TAKEN	MATERIAL EXAMINED				POLARIMETR	
				SAMPLE	DATE	SOURCE	CRYST. OR TOTAL	Mg. % IN $CHCl_3$	NET READING IN DEGREE
52	W. B. M 75	Pneumonia, coronary disease	1/56, T 2/57, P	a	7/29/57	Cecum	T	2.25	+0.072
				b	7/29/57	Sigmoid	T	1.5	+0.055
53	C. P. M 55	Coronary disease, alcoholism	None known	a	7/31/57	Cecum	T	3.8	-0.130
				b	7/31/57	Sigmoid	T	6.25	-0.236
54	C. O. F 3	Killed, traffic accident	None known	a	7/30/57	Sigmoid	T		
55	S. E. M 13	Accidental death	None known	a	8/27/57	Cecum	T	3.09	+0.010
				b	8/27/57	Sigmoid	T	2.25	-0.056
56	J. W. M 78	Death due to CO	None known	a	8/16/57	Cecum	T	2.15	-0.065
				b	8/16/57	Sigmoid	T	3.37	-0.092
57	M. C. M 34	Anoxia, asthma, acute bronchitis	None known	a	9/ 5/57	Cecum	T		
				b	9/ 5/57	Sigmoid	T		
58	D. P. M 17	Accidental death	None known	a	9/11/57	Cecum	T	3.6	-0.104
				b	9/11/57	Sigmoid	T	4.0	-0.130
59	C. K. F 52	Acute coronary occlusion	None known	a	10/16/57	Cecum	T	2.95	-0.094
				b	10/16/57	Sigmoid	T	3.7	-0.137
60	J. S. M 74	Sudden death	None known	a	10/15/57	Cecum	T		
				b	10/15/57	Sigmoid	T		
61	J. H. M 32	Coronary occlusion	None known	a	10/16/57	Cecum	T		
				b	10/16/57	Sigmoid	T		
62	B. H. F 65	Carcinomatosis	4/19/56 P, T, S	a	6/28/56	Bile	T	2.76	+0.004
				b	7/ 3/56	Bile	T	8.1	-0.258
				c	7/ 8/56	Bile	T	4.08	-0.073
63	J. K. M 74	Hemolytic anemia	None	a	12/ 7/55	Duodenal contents	T	2.15	-0.044
				b	12/ 7/55	Duodenal contents	C	1.58	-0.057
64	T. S. F 50	Cholelithiasis, ch. cholecystitis	6/28/56 P, S	a	7/ 6/56	Bile	T		
65	E. M. M 66	Cholecystectomy, ch. cholecystitis	9/27/56 P, S	a	10/ 5/56	Bile	T		
66	A. K. M 72	Subtotal gastrectomy cholecystectomy	7/3/56 P, S	a	7/ 7/56	Bile	T		
67	C. W. M 27	Hemolytic anemia	7/19/53 T	a	11/ 3/55	Duodenal contents	T		
68	L. M	Cholecystectomy, T-tube drainage		a	8/23/55	Bile	T		
				b	9/20/55	Bile	T		

*P = penicillin; S = streptomycin; E = erythromycin; T = tetracycline; F = furadantin; Su = sulfonamide; N = neomycin.

†Insignificant or doubtful (see Table I).

—CONT'D

$[\alpha]^{20}_{D}$	CASE AND SAMPLE NO.	μg TOTAL U GROUP OXID. $FeCl_2$	ABSORPTION: PER CENT ABSORPTION mμ 492	560	650	ABSORPTION: 492 / 560 + 650	ABSORPTION: 560 / 650	APPROXIMATE PER CENT OF d, i, l FORMS FROM ABSORPTION RATIOS (FIG. 4) d	i	l	COLOR OF FINAL SOLUTION	μg l-s IN RESID. AQUEOUS
+3,200	52a	200	30	67	86	0.19	0.78	40	60		Blue	4
+3,666	52b	200	12	29	38	0.18	0.76	50	50		Pale blue	3
-3,370	53a	200	33	7	3	3.3	2.3		5	95	Pink	26
-3,619	53b	200	54	9	3	4.5	3.0			100	Pink	38
	54a	200	26	48	44	0.28	1.09		75	25	Purple	4
+324	55a	200	59	79	42	0.487	1.88		85	15	Purple	32
-2,488	55b	200	86	91	15	0.81	6.07		30	70	Purple	96
-3,023	56a	200	86	52	11	1.36	4.73		15	85	Purple	58
-2,730	56b	200	86	91	15	0.97	7.7		20	80	Purple	61
	57a	200	27	62	89	0.18	0.7	65	35		Blue	4
	57b	101	12	27	43	0.17	0.63	75	25		Blue	4
-2,890	58a	200	56	54	9	0.89	6.0		25	75	Purple	62
-3,250	58b	200	87	11	2	6.7	5.5			100	Pink	55
-3,108	59a	200	75	36	5	1.83	7.21		10	90	Pink-purple	103
-3,702	59b	200	52	13	2	3.47	6.5			100	Pink	90
	60a	200	18	49	93	0.12	0.52	100			Aqua	2
	60b	200	43	82	78	0.27	1.05		75	25	Blue-purple	8
	61a	200	54	32	4	1.5	8.0		10	90	Pink-purple	74
	61b	200	37	7	2	4.11	3.5			100	Pink	65
+140	62a	200	56	44	9	1.05	4.9		25	75	Pink-purple	47
-3,169	62b	200	66	41	16	1.16	2.5		20	80	Pink-purple	52
-1,789	62c	200	55	47	11	0.95	4.2		25	75	Purple	53
-2,047	63a	200	58	67	13	0.73	5.1		35	65	Purple	48
-3,068	63b	200	51	40	5	1.13	8.0		20	80	Pink-purple	75
	64a	70	33	31	12	0.77	2.6		30	70	Purple	19
	65a	200	41	11	3	2.9	3.6			100	Pink	53
	66a	90	21	43	10	0.396	4.3		70	30	Purple	14
	67a	158	56	14	1	3.72	14.0			100	Pink	35
	68a	200	83	14	3	4.9	4.6			100	Pink	
	68b	200	58	32	2	1.7	16.0		40	60	Pale purple	33

as in No. 14b, or too great as in the remaining 3. In two instances (Nos. 55 and 62) the values were significant but in the wrong direction, while in two others (Nos. 11b and 49) the values were insignificant according to the analysis in Table I. In both of these, however, strong dextrorotation would be required to agree with the $FeCl_3$ results. These discrepancies are unexplained but the possibility must be considered that other compounds are represented, such as Gray's racemic dehydrourobilin,[50] or dextrorotatory isomer of i-urobilin,[50] or a complex of any 2 of the 3 ordinary types, with resultant change of optical activity. Since the $FeCl_3$ method permits comparison of final color with the spectral distribution curve and the ratios used (Fig. 4), it is regarded as generally more reliable, especially where 200 μg of starting urobilin group was oxidized.

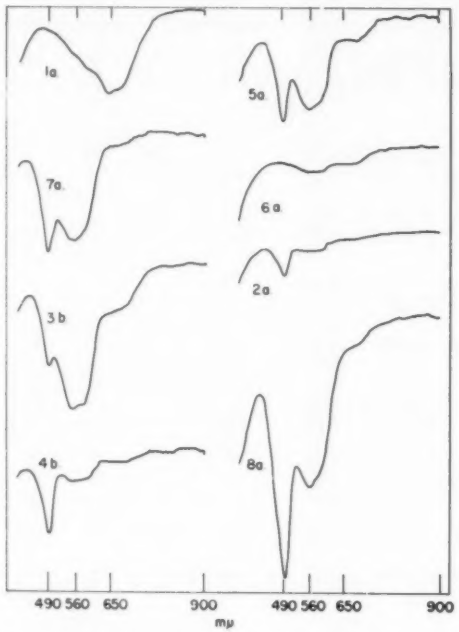

Fig. 5.—Spectral distribution curves after $FeCl_3$ oxidation of urobilin group from normal urines (the individual numbers, e.g., 1a, relate to Table II). Beckman DK spectrophotometer, drive 10; optical density increasing from above downward.

It is evident that normal urine contains significant and at times preponderant amounts of i-U (Table II, Cases 1 to 8 inclusive). This is contrary to the belief that only l-S is present normally.[28, 31, 32] d-U was encountered but once in the normal group (Case 1) and this individual had had antibiotics, type undetermined, 8 months earlier. Illustrative spectral distribution curves are shown in Fig. 5. The feces from Patient S. G. (Case 7) contained varying amounts of i-U. As seen in Table II, i-U was preponderant in the first sample

studied (7a), one year after erythromycin, while samples 7b and c, 3 months later, contained mainly l-S. It is unnecessary to postulate that the preponderant i-U in sample 7a was related to the erythromycin, as it is seen that i-U is often encountered, both in feces and urine, where there was no history of antibiotic intake. The same is true, although less frequently, of the d-form. In Case 14 both urine and feces contained only d-U. This individual had received penicillin which in many other instances did not affect the normal preponderance of l-S. Furthermore, it is evident that in cases of sudden death, with no recent illness and no history of antibiotics, the cecum and sigmoid at times contained d-U. Case 60 is of special interest in this regard, as the cecum contained only the d- form, and the sigmoid contained 75 per cent i- and 25 per cent l-. In Case 57 essentially the same proportions of d- and i- were found in cecum and sigmoid, the values actually indicating a slightly larger proportion of d-U in the sigmoid contents. While the difference is perhaps insignificant it is possible that d-U formation was still in process in the sigmoid. As will be reported elsewhere, the normal feces regularly contain small amounts of bilirubin, hence any of the compounds along the pathway of reduction to stercobilinogen might also be encountered. The data from cecum and sigmoid indicate, however, that in the main the reduction to i-U, and at times to l-S, is largely completed in the cecum. In certain instances, as in Case 55 (Table II), the progression from one to the other in cecum and sigmoid is strongly supported.

No consistent differences were observed in respect to the proportion of the 3 members of the urobilin group, as between hepatic and hemolytic states. It is evident in Table II that various proportions of d-, i-, and l- forms are encountered in both categories of disease. Insofar as the urine in hemolytic states is concerned, it is also clear that d-U is often present, despite lack of history of antibiotic intake. In Case 26 (Table II) the urine contained only the d- form at a time shortly after the feces were found to contain 80 per cent i- and 20 per cent l-. This is explicable on the basis of formation and reabsorption of d-U from the right colon at the time represented by the urine collection; the fraction not absorbed being reduced to i-U and l-S in further traversal of the colon and prior to excretion in the feces. As might be anticipated from our earlier observations, the occurrence of d-U is more often associated with recent use of broad spectrum antibiotics. The observation that d-U is at times the sole representative of the urobilin group in the feces, even where there was no history of antibiotic intake, as in Case 14 (sample b), provides strong support for the enterogenous nature of all members of the urobilin group, especially as it is clear from studies described elsewhere[1c] that the normal fecal flora in broth cultures readily reduces bilirubin (probably as glucuronide) to d-, thence to i- and l- forms.

Table III includes the urinary and fecal data from a case of hepatic cirrhosis receiving broad spectrum antibiotics intermittently. It is seen that d-U is consistently preponderant except after discontinuance of tetracycline when i-U is prominent and small amounts of l-S appear. In other cases of liver

disease, either hepatitis or cirrhosis (Table II), the urine contained i- and l-forms in varying proportion, rarely d-U even without history of antibiotic intake. Illustrative spectral distribution curves are shown in Fig. 6.

Variations in the composition of the bile urobilin group may be noted in Table II (Cases 62 to 68 inclusive). Illustrative spectral distribution curves from these samples are shown in Fig. 7. It is evident in Table II that the l-form (stercobilin) is usually preponderant even with sterile samples, as in

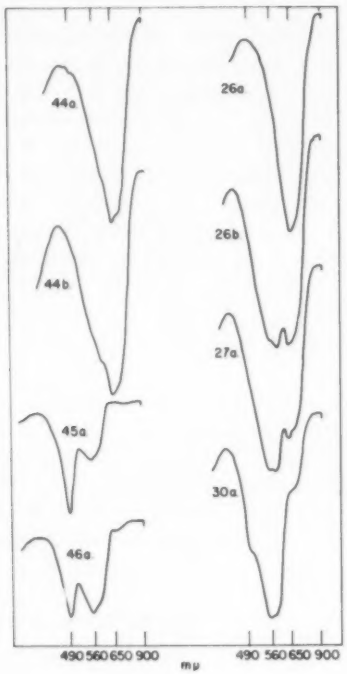

Fig. 6.

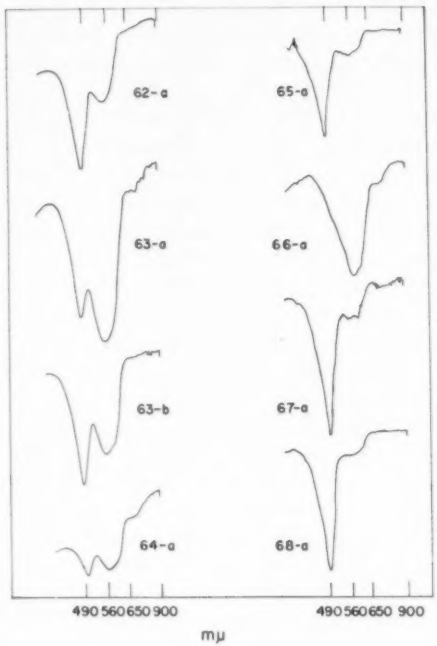

Fig. 7.

Fig. 6.—Spectral distribution curves after $FeCl_3$ oxidation of urobilin group from urines of patients with liver disease. (The individual numbers, e.g., 44*a*, relate to Table II.) Beckman DK spectrophotometer drive 3; optical density increasing from above downward.

Fig. 7.—Spectral distribution curves after $FeCl_3$ oxidation of urobilin group from various samples of bile (the individual numbers, e.g., 62*a*, relate to Table II). Beckman DK spectrophotometer, drive 3; optical density increasing from above downward.

Case 68. The sterile sample from Case 66 contained a majority of the i- form, while contrary to the usual finding in previous studies, the heavily overgrown sample from Case 62 contained mainly l-S rather than d-U. This, however, is quite compatible with our more recent observations that efficient bacterial reduction readily converts d- to i- and i- to l-. l-S was also preponderant in the urobilin group obtained from the duodenal contents of the two cases of hemolytic anemia (Cases 63 and 67).

TABLE III. VARIATIONS IN UROBILINOGEN GROUP DURING LONG PERIOD OF ANTIBIOTIC THERAPY IN PATIENT G. R. (A 35-YEAR-OLD MAN WITH CIRRHOSIS)

ANTIBIOTICS	DATE	MATERIAL STUDIED	RESULTS OF $FeCl_3$ OXIDATION (200 μg U GROUP)								
			COLOR FINAL 5% HCl	BECKMAN ABSORPTION (100 % TRANS.)			492 (l-s) 560 + 650	560 (Mv-Mr) 650 (gl)	APPROXIMATE PER CENT		
				492	560	650			d	i	l
Tetracycline started	8/19 to 20/55	Urine	Aqua	14	47	90	0.102	0.52	100		
	8/22 to 23/55	Urine	Aqua	10	27	73	0.10	0.36	100		
	8/20/55	Feces	Aqua	14	20	89	0.12	0.22	100		
	8/21/55	Feces	Aqua	17	46	91	0.12	0.50	100		
	8/22/55	Feces	Aqua	16	50	93	0.11	0.55	90	10	
Penicillin and streptomycin continued 8/24 thru 9/16	8/24/55	Feces	Aqua	14	46	80	0.11	0.58	90	10	
	8/25 to 26/55	Urine	Aqua	16	50	88	0.12	0.57	90	10	
	8/26/55	Feces	Aqua	14	44	78	0.11	0.56	90	10	
	8/26 to 27/55	Urine	Aqua	14	38	79	0.12	0.48	100		
Tetracycline discontinued	8/27/55	Feces	Aqua	16	57	92	0.10	0.61	90	10	
	8/28/55	Feces	Aqua	17	56	92	0.12	0.60	90	10	
	8/28 to 29/55	Urine	Aqua	15	41	88	0.12	0.47	100		
	8/29/55	Feces	Aqua	15	52	89	0.11	0.58	90	10	
Tetracycline started	8/30/55	Feces	Aqua	21	56	88	0.15	0.63	85	15	
	9/ 3/55	Feces	Blue	48	80	76	0.30	1.05		75	25
	9/ 7/55	Feces	Blue	31	64	83	0.21	0.77	60	40	
Tetracycline discontinued	9/11 to 12/55	Urine	Blue	28	67	87	0.11	0.77	60	40	
	9/13/55	Feces	Purple	55	82	60	0.38	1.36		60	40
	9/13 to 14/55	Urine	Blue	54	78	61	0.39	1.27		60	40
	9/18 to 19/55	Urine	Aqua	21	56	88	0.15	0.61	85	15	
	9/20 to 21/55	Urine	Aqua	13	38	90	0.1	0.42	100		
	9/24 to 25/55	Urine	Blue	30	68	88	0.19	0.77	60	40	
	9/27 to 28/55	Urine	Purple	46	75	67	0.32	1.11		60	40
	10/ 3 to 4/55	Urine	Purple	44	79	72	0.29	1.09		80	20
	10/ 6 to 7/55	Urine	Blue	36	79	85	0.21	0.93		100	

DISCUSSION

The $FeCl_3$ oxidation method, as presently employed, is believed to provide a reasonably exact means of differential quantitative determination of the naturally occurring l-, i-, and d- forms of the urobilin group. The fact that i-U yields variable amounts of glaukobilin, together with mesobiliviolin and mesobilirhodin, prevents a strict separation from d-U, the latter being converted mainly to glaukobilin, under the conditions described (Figs. 2 and 3). Pure mesobiliviolin is converted in large part to glaukobilin by the same $FeCl_3$ oxidation procedure. It is clear from the present results that the glaukobilin is the same, whether from i- or d-U.

While there is some overlap of spectral distribution after $FeCl_3$ oxidation of i- and d-U (Figs. 2 and 3), the combined information gained by the color of the final 1.5 N HCl solution, the character of the spectral distribution curve, especially the presence of absorption in the 560 mμ region, and consequently the $\frac{560}{650}$ ratio, and the optical activity measurement permit reliable conclusions as to the approximate proportions of the two compounds.

The data provided by these methods for feces, urine, and bile, clearly support the enterogenous character of all 3 members of the urobilin group,

d-U, i-U, and l-S. It must be emphasized again that these are not isomers in any sense and that the designations are used solely for convenience and simplicity in discussing 3 closely similar substances historically grouped under the name urobilin.

The present results fail to support Baumgärtel's dichotomous concept[28] of the origin of the urobilins, nor do they confirm the reports[29-34] that there are significant or consistent differences between hemolytic states on the one hand, and those of the hepatic injury on the other. The belief that stercobilin (l-S) alone is normally present in the urine is not borne out by the present findings which reveal the occurrence of variable amounts of i-U and even of d-U. While it is true that the latter is encountered much more frequently after broad spectrum antibiotics it is highly significant that in the present study it has been observed in urine samples and in cecal contents of subjects without history of antibiotic intake. The simultaneous occurrence of d-U in the cecum and i-U in the sigmoid clearly indicates the progression of bacterial reduction along the colon. This could scarcely be reconciled with Baumgärtel's concept that i-U is formed only in the liver or biliary tract. The data on cecal and sigmoid contents in Table II reveal considerable variation in the type represented, probably on the basis of variable efficiency of reduction by the fecal flora. This is quite in accord with recent observations on urobilinogen formation by fecal bacteria, in vitro, as discussed elsewhere.[1c] These studies have shown that in broth cultures the normal fecal flora (1) often reduces bilirubin to d-U, with smaller amounts of i-U and l-S, and (2) readily reduces d-U to i-U and l-S, and i-U to l-S. In contrast, the chemical, nonenzymatic reduction of d-U proceeds only to the i-form.

Thus the finding of d-U with little or no i-U and no demonstrable l-S in feces from individuals receiving broad spectrum antibiotics strongly indicates that the effect of the latter is simply to halt bacterial reduction at the d-stage, preventing the normal further reduction to i-U and l-S. The pattern of d- and i- excretion is well demonstrated in Table III, a case of special interest in that the patient had advanced cirrhosis of the liver and was receiving tetracycline intermittently. In addition to the antibiotics shown in the table the patient had previously received tetracycline, which may account, at least in part, for the d-U in the first samples shown. Baumgärtel[30] postulated that the broad spectrum antibiotics injure the liver in a fashion which prevents the alleged hepatic formation of mesobilirubinogen. According to this assumption, certain types of hepatic injury, such as infectious hepatitis, promote the formation of i-U, while others, such as that ascribed to tetracycline, prevent it. The data in Table III are much more readily understood as a varying inhibition of intestinal bacterial reduction, being greater when d-U alone was noted, less when i-U and l-S were also present.

It is evident too that even in the absence of demonstrable hepatic impairment, as in some cases of hemolytic jaundice (Cases 26 to 28 in Table II) and in some normal individuals, i-U may predominate in the feces. Furthermore,

in a study* with G. Ugarte, it was found that i-U is at times the main representative of the urobilin group in the feces and urine of the normal dog. In a still earlier study with Sborov and Schwartz,* d-U was encountered on occasion in normal dog feces.

The various observations on feces, bile, and urine, especially when considered in relation to those on cecal and sigmoid contents, are only compatible with an enteric derivation of the urobilin group, i.e., reduction of bilirubin by the intestinal flora.

The presence of members of the urobilin group and especially of l-S in samples of sterile fistula bile (Case 68, Table II) and in the duodenal contents of patients with hemolytic anemia confirms the concept of an enterohepatic circulation, but leaves undetermined the extent to which the reabsorbed members of the urobilinogen group are altered or destroyed by the liver.

Some further comment is needed as to the place of d-U in the normal stepwise bacterial reduction to bilirubin in the human colon (see Fig. 8).

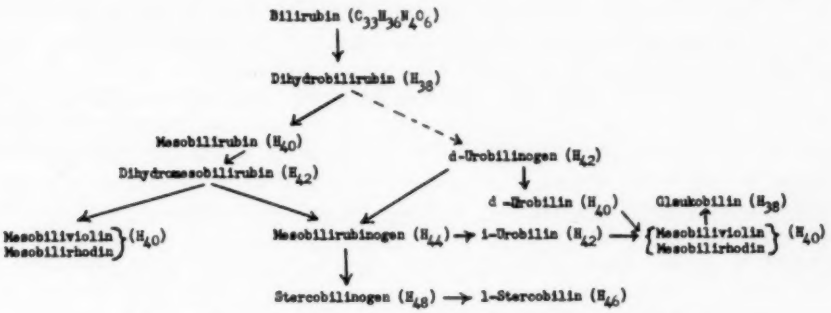

Fig. 8.—Relationships of principal naturally occurring bilirubinoids.

Evidence has been presented in the foregoing that it does occur under normal circumstances. Elementary analyses of crystalline d-urobilinogen (d-Ubg)[46, 47] revealed that it is isomeric with dihydromesobilirubin ($C_{33}H_{42}N_4O_6$) which is known to be formed as an intermediary in the catalytic hydrogenation of mesobilirubin to i-Ubg.[48] Dihydromesobilirubin is light yellow in color; d-Ubg is colorless.[46, 47] Dihydromesobilirubin is rapidly converted to mesobiliviolin under the same conditions yielding d-U from its corresponding urobilinogen. No evidence of urobilin formation from dihydromesobilirubin has been obtained. This possibility was suggested by Meyer.[49] Evidence was obtained in a study by Lowry and Watson[47] that small amounts of mesobilirubin and dihydromesobilirubin occur in normal feces. In a study to be described elsewhere, dihydromesobilirubin was found in human cecal contents in association with relatively large amounts of d-Ubg which was readily isolated in crystalline form. While it is clear that the two isomers are both

*Unpublished.

formed by bacterial reduction of bilirubin in the colon and that both are ordinarily converted by further reduction to i-Ubg (mesobilirubinogen) and thence to l-Sbg (see Fig. 8) their relationship and the point of origin of i-Ubg along the pathway from bilirubin remain in doubt. It is possible that d-Ubg is formed either (1) by rearrangement of dihydromesobilirubin, (2) by an independent asymmetric reduction of mesobilirubin, or (3) by a reduction of dihydrobilirubin in which a single vinyl group is left intact (see below). Siedel[16] has suggested that formation of both d-Ubg and l-Sbg depends on a primary formation of tautomers of i-Ubg (mesobilirubinogen), tautomer "A" being the precursor of another intermediary tautomer "B," which he regards as the precursor of d-Ubg. The formula suggested by Siedel for d-U is at variance with that recently proposed by Gray and Nicholson.[50] The latter authors have described evidence indicating that d-U may retain one of the vinyl groups of bilirubin. In this respect the molecule would then be similar to dihydrobilirubin, a monovinyl, monoethyl compound representing the first stage in the hydrogenation of bilirubin. The bacterial reduction of dihydrobilirubin might bypass mesobilirubin and dihydromesobilirubin to produce d-Ubg, and it is quite conceivable that the latter could be formed in this way in larger amount than are the meso-compounds. It might seem that the preponderant formation of glaukobilin by dehydrogenation of d-U would exclude the presence of a vinyl group, since glaukobilin has two ethyl groups and, in other words, is a meso- derivative. Gray and Nicholson[50] have postulated rearrangement or hydrogen transfer by which the vinyl group could be converted into an ethyl group, without addition of hydrogen to the molecule. If the Gray-Nicholson formula for d-U is confirmed, the pathway of urobilinogen formation in the colon will be as diagrammed in Fig. 8. From this it is evident that d-Ubg could not then be regarded as an obligatory precursor of the i- and l- members of the urobilinogen group. At present, a principal objection to the structure proposed by Gray and Nicholson[50] for d-U is the infrared spectral identity[46] with i-U in which there is no vinyl group. The structure proposed by Siedel,[16] in its lactam form, is in better agreement at least from this standpoint. This structure would indicate derivation of d-Ubg from meso- or dihydromesobilirubin, rather than dihydrobilirubin as suggested in Fig. 8, based on the evidence of Gray and Nicholson. Although in the present study d-U has been found under apparently normal conditions, it is possible that unrecognized variations in the intestinal flora might shift the pathway in either of the directions shown (Fig. 8).

The differing formation of mesobiliviolin and glaukobilin in the $FeCl_3$ method upon which the present results depend has been the subject of a detailed study to be described separately.

The composition of the urobilin group in urine, bile, and feces at any one time thus appears to depend on an interplay of several factors operative in the colon, i.e., the efficiency of bacterial reduction, the degree and site of absorption depending on filling of the colon, and the motility of the intestinal contents. The local formation of the urobilinogen group by bacterial activity

in the biliary[27] or urinary tracts, while relatively unimportant, must be recognized as an occasional event. In the main the urobilin(ogen) group of urine, bile, and feces, is formed in the colon, quite in accord with the early views of Maly, von Müller, and McMaster and co-workers.

SUMMARY AND CONCLUSIONS

1. The composition of the urobilin(ogen) group in urine, bile, and feces has been studied under normal and a variety of pathologic circumstances, especially in hepatic and hemolytic states.

2. The approximate proportion of the 3 members of the group, d-U, i-U, and l-S was determined by suitable fractionation, spectrophotometry, and colorimetry, following ferric chloride oxidation. Measurement of optical activity was of supplementary value, although not always in agreement.

a. Stercobilin (l-S) is relatively stable to $FeCl_3$ oxidation in the sense that it does not yield mesobiliviolin-mesobilirhodin or glaukobilin, and a considerable fraction is unchanged; under the conditions employed i-U is converted to a mixture of mesobiliviolin-mesobilirhodin and glaukobilin, and d-U mainly to glaukobilin. The glaukobilin obtained from d-U and i-U has been crystallized and shown to be identical. The ratio of absorption at $\frac{492\ m\mu}{560 + 650\ m\mu}$ permits determination of the proportion of l-S as contrasted with i-U + d-U; that of the absorption at $\frac{560\ m\mu}{650\ m\mu}$ relates to the proportion of i- and d- in the original urobilin sample.

b. Optical activity measurement is of principal value in confirming the presence of d-U when accompanied by considerable amounts of i-U.

3. Variations in composition were observed without reference to any particular state of health or disease. i-U was preponderant in the urine of certain normal individuals, l-S (stercobilin) in others; the same was true both in hemolytic and hepatic diseases. The fecal urobilin composition was likewise found to vary widely; in certain individuals i-U was preponderant or almost solely represented. In accordance with previous reports administration of broad spectrum antibiotics is followed by a great preponderance of d-U in feces, urine, and bile. d-U is also observed at times where antibiotics have not been used; it has been found repeatedly in the contents of the cecum of normal individuals, the sigmoid colon at the same time usually containing greater proportions of i-U or l-S.

4. These findings are only compatible with an enteric source of all 3 members of the urobilin(ogen) group. It is believed that d-U is a normal product of the bacterial reduction of bilirubin, but its exact point of origin in the pathway has not been determined. The variations in composition of the urobilinogen group from time to time are believed to be based on factors of bacterial activity and intestinal absorption.

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VENTILATORY RESPONSE TO INCREASING ARTERIAL CO_2 TENSION EMPLOYING A REBREATHING METHOD IN NORMAL INDIVIDUALS AND IN PATIENTS WITH CARDIAC DISEASE

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ALTHOUGH carbon dioxide tension in the arterial blood (Pa_{CO_2}) is usually considered to be the major stimulus to respiration in the normal individual, various investigators have demonstrated that this situation may be modified in certain disease states. In particular, a diminished ventilatory response to increased CO_2 in the inspired air has been universally reported in chronic obstructive emphysema with hypercapnia.[1, 2] On the other hand, the reverse situation has been demonstrated by Julich[4, 5] in certain patients with cardiac disease, in whom the ventilatory response to an elevation of Pa_{CO_2} may be greater than normal. In these studies, patients with mitral disease differed, however, by exhibiting a relatively low, or even a decreased, ventilatory response.

The present investigation was undertaken to study the ventilatory response to elevated Pa_{CO_2} in normal individuals and in patients with cardiac disease, and to determine whether the findings in the latter are altered after cardiac surgery for correction of the defect involved. Although previous investigators in this field have measured the response to a known concentration of CO_2 in the inspired air, we chose to employ a modification of Julich's rebreathing method,[3] correlating total ventilation with arterial CO_2 tension. This was done for the following reasons:

1. Correlation of Pa_{CO_2} measurements with the ventilatory response permits direct quantitation of the physiologic stimulus with the effect it produces. Even in normal individuals it is unsafe to assume that a given inspired CO_2 will produce predictable arterial CO_2 tensions, since respiratory quotient, ventilatory rate, impaired intrapulmonary gas distribution, altered ventilation-perfusion ratios, and venous admixture due to shunts are inconstant variables.

2. Although effective alveolar ventilation has been used by some as a measurement of the ventilatory response,[1, 2] evidence is available which indicates that the respiratory centers respond by increasing *total* ventilation.[6]

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3. Finally, the rebreathing technique permits a gradual elevation in Pa_{CO_2} and ventilation, permitting measurements to be made at any selected point of ventilatory response.

In analyzing the data on relationship of Pa_{CO_2} to ventilatory response, it is essential to draw a careful distinction between the terms "threshold" on the one hand, and "sensitivity" or "responsiveness" on the other. Gray[7] clearly defies these when he states that: "These two fundamental aspects are: (a) the absolute magnitude of a stimulus, which produces a just detectable response, and (b) the increment in response produced by a given increment in stimulus. The first of these is the *threshold* phenomenon, and the second is *sensitivity* or irritability." For the purpose of this study, "sensitivity" or "responsiveness" of the respiratory center is defined as the increase in ventilation in liters per minute produced by 1 mm. Hg increase in Pa_{CO_2}, as expressed by the relationship, $\frac{\Delta \dot{V}}{\Delta Pa_{CO_2}}$, hereafter referred to as the "*quotient of responsiveness*" (QR). Previous investigators have shown this relationship to be linear within the range of physiologic stimulation and response.[1, 2, 7, 8] The "threshold," or theoretical point of apnea, on the other hand, is defined as the level of Pa_{CO_2} at and below which this stimulus is presumably ineffective in producing any ventilatory response. Thus, if Pa_{CO_2} remained constant, an increase in ventilation could represent either an increased responsiveness to the CO_2 stimulus, or a lowering of the threshold.

MATERIALS AND METHODS

Material for this study comprised a total of 31 individuals divided into 4 groups as follows:

Group I: Eleven normal persons selected from hospital personnel and patients in whom cardiorespiratory disease was excluded by history and physical examination.

Group II: Nine patients with rheumatic heart disease consisting of "pure" or predominant mitral stenosis.* Since clinical, physiologic,[21] and pathologic[23] observations indicate that pulmonary changes in mitral stenosis occur only after a number of years have elapsed, the group was arbitrarily divided into those whose active rheumatic fever had occurred more than 10 years (7 patients) and less than 10 years (2 patients) previously.

Group III: Four patients with rheumatic heart disease whose lesions were predominantly aortic.* Three of these exhibited signs of mitral involvement as well.

Group IV: Seven patients with congenital heart disease of several types. Cardiac catheterization, performed in each of these individuals, revealed varied hemodynamic derangements. Patients D. K. and J. C., with a patent ductus arteriosus and pulmonic stenosis, respectively, exhibited no pulmonary hypertension. In Patients I. M. and I. B., borderline pulmonary hypertension was found, while the remaining 3 patients showed considerable to extreme pulmonary hypertension. Slight arterial oxygen unsaturation during 100 per cent oxygen inhalation in Patients I. M. and I. B. suggested a right-to-left shunt, possibly on the basis of undetected pulmonary atrioventricular communications.

*In one patient of Group II and 3 patients of Group III, left heart catheterization was performed by Dr. E. H. Drake of the Department of Adult Cardiology.

Several patients were under treatment with digitalis and mercurial injections at the time of the study. None had been receiving ammonium chloride, diamox, aminophylline, or other drugs known to affect the respiratory center. A summary of the diagnoses and pertinent findings in all 20 patients is presented in Table I.

TABLE I. CLINICAL DATA ON ALL PATIENTS

NAME	AGE	CLINICAL FINDINGS CONSISTENT WITH:	AMERICAN HEART ASSOCIATION CLASSIFICATION	CONGESTIVE FAILURE
Group II. Pure or Predominant Mitral Stenosis (9 Patients)				
		A. Less than 10 years since onset of rheumatic fever (2 patients)		
1. E. B.*	16	Pure mitral stenosis	II	0
2. L. B.	13	Pure mitral stenosis	I	0
		B. More than 10 years' duration since rheumatic fever (7 patients)		
3. R. U.	51	Mitral stenosis and mitral insufficiency	III	+
4. E. E.	52	Mitral stenosis and mitral insufficiency	III	+
5. I. C.	46	Pure mitral stenosis	II	0
6. P. K.	28	Pure mitral stenosis	II	0
7. H. E.*	43	Pure mitral stenosis	III	0
8. I. S.*	49	Pure mitral stenosis	II	0
9. G. K.*	32	Pure mitral stenosis	II	0
Group III. Predominantly Aortic Lesions (4 Patients)				
1. J. R.	45	Aortic stenosis, mitral stenosis, and mitral insufficiency	III	0
2. G. B.*	43	Aortic stenosis and mitral stenosis	III	+
3. D. B.*	42	Aortic stenosis, aortic insufficiency, mitral stenosis, and tricuspid insufficiency	IV	+
4. P. J.*	38	Aortic stenosis	II	0
Group IV. Congenital Lesions (7 Patients)				
1. D. K.	23	Patent ductus arteriosus	I	0
2. E. N.	51	Patent ductus arteriosus	III	+
3. S. M.	23	Interventricular septal defect	III	+
4. D. H.	22	Interatrial septal defect	I	0
5. J. C.	33	Pulmonic stenosis	II	0
6. I. M.	38	Interatrial septal defect	III	+
7. I. B.	31	Patent ductus arteriosus	II	0

*Pre- and postoperative studies done.

Technically satisfactory postoperative studies after corrective cardiac surgery were performed in 7 patients, 4 in Group II and 3 in Group III. In Patient H. E. (Group II) the operation was unsuccessful because of the development of postoperative mitral insufficiency. Since he demonstrated obvious hemodynamic abnormalities persisting after surgery, his findings are listed separately in the results. Three patients in Group IV underwent cardiac surgery, but none have been studied postoperatively. Preoperative diagnosis was confirmed in all patients at the time of surgery.

The procedure, as modified from Julich,[8] required the seated patient to breathe 100 per cent oxygen* from a 100 L. reservoir bag, introduced into a closed system with no device for removal of carbon dioxide. By means of appropriate valves and an outlet to the room, the subject first breathed 100 per cent oxygen in an open circuit for 20 to 30 minutes in order to stabilize respiration and blood gases.[9] On closing the circuit, a very gradual accumulation of carbon dioxide occurred within the system, reaching a concentration of approximately 6 per cent in the reservoir bag within 40 to 60 minutes.

*Oxygen is used instead of room air to ensure full arterial oxygen saturation throughout the test, thereby eliminating possible anoxic stimulus by the chemoreceptors. In addition, the experiment was better tolerated by cardiac patients, for whom the desired levels of hyperventilation constituted considerable exertion.

Arterial blood samples, using an indwelling Riley needle in the brachial artery, were obtained at the end of the initial oxygen inhalation period, and at 3 points during the rebreathing period, the last at a level approximately 3 times that of the resting ventilation. Minute ventilation was calculated from 3-minute readings from a gasometer (previously calibrated against a Tissot spirometer) introduced on the inspiratory side of the circuit. In view of the fact that *change* in *total* ventilation was the measurement required, and that all tests were performed in an air-conditioned room with a mean temperature of 23° ± 0.2° C., no corrections for temperature or pressure were applied. The maximum temperature rise in the circulating gases of the system at the end of the experiment was 0.3° C.

The attainment of a complete steady state during the rebreathing procedure was considered unnecessary in view of the gradual increase in CO_2 concentration and the comparative nature of the results.

Arterial blood oxygen content, capacity, and CO_2 content were determined in the Van Slyke manometric apparatus. Arterial blood pH was measured in a Cambridge glass electrode at a temperature of 37.5° C. Values for Pa_{CO_2} were calculated from the Singer-Hasting nomogram[10] with appropriate corrections for hematocrit values.

Stimulus-response curves obtained from these data confirmed the linear relationship between total ventilation and arterial P_{CO_2}. Measurement of 4 separate points afforded an additional check on technical error, which would be suspected in any determination showing a gross deviation from an ideal straight line. The increase in total ventilation per millimeter of rise in Pa_{CO_2}, or quotient of responsiveness (QR), could then be calculated from the slope of the curve. Values for QR per square meter of body surface ($QR/M.^2$) were used in order to provide more accurate comparative data. The point at which the curve intersects the abscissa represents the threshold value for this stimulus, i.e., the theoretical highest level of Pa_{CO_2} which would produce no ventilatory response.

TABLE II. LABORATORY DATA IN 11 NORMAL INDIVIDUALS

NAME	AGE (YEARS)	BODY SURFACE AREA ($M.^2$)	VENTILATION* (L./MIN./$M.^2$) START	VENTILATION* (L./MIN./$M.^2$) END	Pa_{CO_2}* (MM. HG) START	Pa_{CO_2}* (MM. HG) END	Pa_{CO_2} THRESHOLD (MM. HG)	$QR/M.^2$
1. H. P.	32	1.92	4.90	20.7	38.8	50.3	33.0	0.94
2. O. C.	38	1.82	3.90	19.8	41.0	64.5	37.0	0.67
3. W. M.	30	1.92	3.91	14.4	37.5	48.0	34.0	1.07
4. S. S.	23	1.78	6.18	16.1	40.0	49.5	36.0	1.19
5. J. M.	37	2.01	4.98	13.0	39.0	48.5	30.0	0.81
6. P. M.	19	1.59	4.59	13.7	40.2	47.0	37.0	1.37
7. J. P.	71	1.80	4.50	14.7	40.8	47.5	38.0	1.57
8. D. P.	31	1.62	5.49	15.0	41.0	49.0	38.0	1.78
9. M. P.	48	1.53	4.18	13.1	42.0	51.5	38.0	1.18
10. H. H.	40	1.77	4.46	11.7	39.4	47.2	33.0	0.78
11. K. D.	31	2.00	4.40	13.9	41.5	51.5	37.0	0.96
Mean	36.6	1.80	4.65	15.1	39.9	50.4	35.5	1.12

*Values recorded for first and last determinations only (see text): Start = breathing 100 per cent oxygen, open circuit; End = third determination while rebreathing 100 per cent oxygen, closed circuit.

RESULTS

Pertinent laboratory data for the 11 normal individuals are presented in Table II, and in Fig. 1 are shown graphically the stimulus-response curves for this and each of the other categories studied. In the normal group, a wide variation occurred in the slope of the curves ($QR/M.^2$ = 0.67 to 1.78 with a mean of 1.12), less so in the threshold values (30.0 to 38.0 mm. Hg, mean =

35.5). One subject (H. P.) was tested twice under apparent identical conditions (mean value only recorded) with little change in QR, but a considerable downward shift in the threshold value, from 39 to 27 mm. Hg. Another apparently normal individual (O. C.) demonstrated a low responsiveness ($QR/M.^2 = 0.67$) with a high threshold (37.0) and a low basal ventilation, suggestive of the decreased responsiveness which has been observed in patients with chronic pulmonary emphysema.[16]

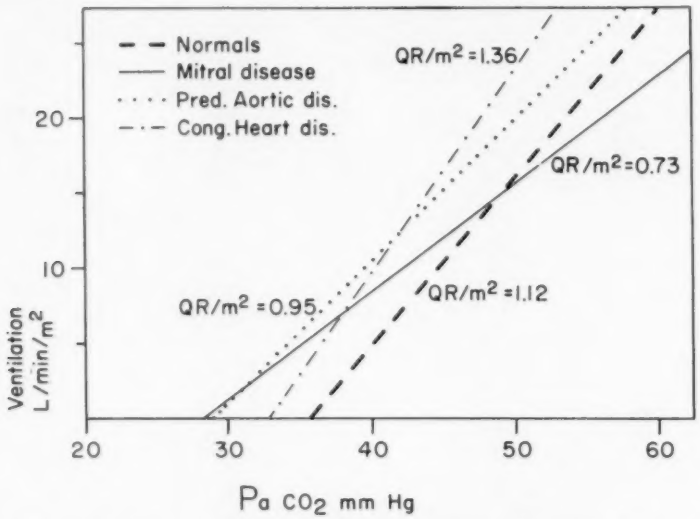

Fig. 1.—Straight lines showing mean relationship between ventilation and Pa_{CO_2} in normal subjects and in 3 groups of cardiac patients.

The results of 4 tests performed in 3 additional persons were excluded from this report for the following reasons. One subject presumed to be normal showed an unusually low responsiveness on two occasions ($QR/M.^2 = 0.64$ and 0.71). Re-examination revealed signs of pulmonary hypertension and pulmonary artery dilatation of yet undiagnosed etiology. One patient with rheumatic heart disease and mitral stenosis, confirmed by left heart catheterization, was found to have severe hypochromic anemia (hemoglobin = 7.0 Gm.) which was thought to alter the central responsiveness. His $QR/M.^2$ was 1.69. One patient with arteriosclerotic heart disease and mild congestive failure showed a responsiveness within the range of the normals ($QR/M.^2 = 0.80$). This type of disorder was not studied further.

Table III presents the same data for the 9 patients with pure or predominant mitral lesions. Among the 7 individuals with disease of more than 10 years' duration, the mean $QR/M.^2$ of 0.73 was significantly lower than that of the normal subjects ($p = < 0.01$). In addition, the mean threshold was considerably lower (28.3 mm. Hg) than that of the normal individuals

(35.5 mm. Hg). Since resting Pa_{CO_2} was not shifted downward a corresponding amount, mild hyperventilation results, amounting to an increase of 0.37 L./min./M.^{2} over the normal.

TABLE III. LABORATORY DATA IN 9 PATIENTS WITH PURE OR PREDOMINANT MITRAL LESIONS

NAME	AGE (YEARS)	BODY SURFACE AREA (M.^{2})	VENTILATION* (L./MIN./M.^{2}) START	VENTILATION* (L./MIN./M.^{2}) END	Pa_{CO_2}* (MM. HG) START	Pa_{CO_2}* (MM. HG) END	Pa_{CO_2} THRESHOLD (MM. HG)	QR/M.^{2}
A. Less Than 10 Years Since Onset of Rheumatic Fever								
1. E. B.†	16	1.66	5.54	17.8	33.5	46.5	30.0	1.05
2. L. B.	13	1.49	6.17	20.6	36.5	47.5	32.0	1.42
B. More Than 10 Years Since Onset of Rheumatic Fever								
3. R. U.	51	1.51	5.22	17.6	32.5	51.5	22.0	0.56
4. E. E.	52	1.60	5.06	10.4	33.0	43.0	23.0	0.52
5. H. E.†	43	1.57	5.10	10.4	39.0	44.5	32.0	0.86
6. I. S.†	49	1.40	5.14	18.0	40.5	56.5	34.0	0.81
7. I. C.	46	1.65	4.76	13.9	37.6	50.2	31.0	0.64
8. G. K.†	32	1.68	5.36	16.2	36.5	52.5	25.0	0.55
9. P. K.	28	1.63	4.48	16.0	35.0	46.0	31.0	1.14
Mean (of Group B only)		1.59	5.02	14.6	36.3	49.1	28.3	0.73

*Values recorded for first and last determinations only (see text): Start = breathing 100 per cent oxygen, open circuit; End = third determination while rebreathing 100 per cent oxygen, closed circuit.

†Patients in whom postoperative studies were done.

In Table IV are listed the data obtained in the 4 patients with predominantly aortic lesions. The mean QR/M.^{2} of this group was 0.95, slightly lower than that of the normal group, although not significantly so ($p = < 0.30$). The mean Pa_{CO_2} threshold was reduced to 28.8 mm. Hg, which on this stimulus-response curve is associated with a ventilation 1.52 L./min./M.^{2} above that of the normals at the level of resting Pa_{CO_2}.

TABLE IV. LABORATORY DATA IN 4 PATIENTS WITH PREDOMINANT AORTIC LESIONS

NAME	AGE (YEARS)	BODY SURFACE AREA (M.^{2})	VENTILATION* (L./MIN./M.^{2}) START	VENTILATION* (L./MIN./M.^{2}) END	Pa_{CO_2}* (MM. HG) START	Pa_{CO_2}* (MM. HG) END	Pa_{CO_2} THRESHOLD (MM. HG)	QR/M.^{2}
1. G. B.†	43	1.73	6.88	19.7	32.0	46.0	26.0	1.01
2. D. B.†	42	1.46	6.51	17.3	35.5	50.0	29.0	0.82
3. J. R.	45	1.58	4.37	15.6	39.5	51.0	34.0	0.99
4. P. J.†	38	1.46	6.92	20.5	31.0	45.4	26.0	0.99
Mean		1.56	6.17	18.3	34.3	48.1	28.8	0.95

*Values recorded for first and last determinations only: Start = breathing 100 per cent oxygen, open circuit; End = third determination while rebreathing 100 per cent oxygen, closed circuit.

†Patients in whom postoperative studies were done.

Table V lists the data in the 7 patients with congenital heart disease. As would be expected in this nonhomogeneous group, a wide range was found in all measurements. The increase in mean QR/M.^{2} to 1.36 is ascribable chiefly to the very high values in Patients I. M. and I. B. (1.97 and 2.65, respectively) both of whom were felt to have a right-to-left shunt Again, a moderate hyperventilation was present at rest (0.58 L./min./M.^{2} greater than the normal subjects) associated with a lowering of the mean Pa_{CO_2} threshold to 32.7 mm. Hg.

In summary, the outstanding finding in the group of 20 cardiac patients, as compared to the normal individuals, was the elevation of resting ventilation associated with a diminished threshold to the CO_2 stimulus in all. Respiratory response to an increasing Pa_{CO_2} (QR) was significantly decreased in the 7 patients with long-standing mitral disease. The mean QR/M.^{2} of the 13 remaining cardiac patients was 1.22, close to the mean normal of 1.12.

TABLE V. LABORATORY DATA IN 7 PATIENTS WITH CONGENITAL LESIONS

NAME	AGE (YEARS)	BODY SURFACE AREA (M.^{2})	VENTILATION* (L./MIN./M.^{2}) START	VENTILATION* (L./MIN./M.^{2}) END	Pa_{CO_2}* (MM. HG) START	Pa_{CO_2}* (MM. HG) END	Pa_{CO_2} THRESHOLD (MM. HG)	QR/M.^{2}
1. D. K.	23	1.69	4.73	16.7	31.5	41.5	27.0	1.20
2. E. N.	51	2.02	3.32	12.3	37.0	52.5	35.0	0.74
3. S. M.	23	1.52	8.49	20.1	34.8	46.7	27.0	1.03
4. D. H.	22	1.79	4.80	12.4	41.0	49.0	35.0	0.93
5. J. C.	33	1.63	5.89	14.7	37.5	45.0	31.0	1.02
6. I. M.	38	1.93	4.51	10.5	37.0	40.0	34.0	1.97
7. I. B.	31	1.66	4.88	17.4	42.0	47.0	40.0	2.65
Mean		1.75	5.23	14.9	37.3	45.8	32.7	1.36

*Values recorded for first and last determinations only: Start = breathing 100 per cent oxygen, open circuit; End = third determination while rebreathing 100 per cent oxygen, closed circuit.

TABLE VI. POSTOPERATIVE LABORATORY DATA (7 PATIENTS)

GROUP	NAME	AGE (YR.)	BODY SURFACE AREA (M.^{2})	VENTILATION* (L./MIN./M.^{2}) START	VENTILATION* (L./MIN./M.^{2}) END	Pa_{CO_2}* (MM. HG) START	Pa_{CO_2}* (MM. HG) END	Pa_{CO_2} THRESHOLD (MM. HG)	QR/M.^{2}
Group I. Mitral Stenosis									
A. Less than 10 yr. since onset of rheumatic fever	1. E. B.	16	1.66	4.94	13.0	38.0	53.5	31.0	0.73
B. More than 10 yr. since onset of rheumatic fever									
1. Successful mitral commissurotomy	2. I. S.	49	1.40	4.14	17.1	42.0	50.0	40.0	1.79
	3. G. K.	32	1.68	4.82	15.1	36.8	43.5	34.0	1.61
2. Unsuccessful mitral commissurotomy	4. H. E.	43	1.57	4.65	12.2	44.0	57.0	39.0	0.71
Group III. Predominantly Aortic Lesions	5. G. B.	43	1.73	6.36	14.9	41.0	55.0	31.0	0.69
	6. D. B.	42	1.46	5.68	14.7	45.0	60.0	37.0	0.67
	7. P. J.	38	1.46	5.89	17.5	37.3	49.0	32.0	0.99

*Values recorded for first and last determinations only: Start = breathing 100 per cent oxygen, open circuit; End = third determination while rebreathing 100 per cent oxygen, closed circuit.

The results of postoperative studies in the 7 patients in whom satisfactory tests were obtained are summarized in Table VI. All showed a postoperative increase in the Pa_{CO_2} threshold, with concomitant reduction of resting ventilation. Change in respiratory response to Pa_{CO_2} (QR) after operation was variable, but the two patients with long-standing mitral stenosis successfully relieved by commissurotomy showed a complete reversal of the mean QR/M.^{2} value from a low figure of 0.68 to an overcorrection of 1.70 (shown graphically in Fig. 2). On the other hand, Patient H. E., whose surgery was unsuccessful because of postoperative mitral insufficiency, exhibited little change in

his QR value after surgery ($QR/M.^2$ = 0.86 preoperatively, and 0.71 postoperatively). Since mitral commissurotomy is not commonly performed in patients with relatively short duration since rheumatic fever, only one such individual (E. B.) could be studied after operation; in this patient, a reduction in $QR/M.^2$ value from 1.05 to 0.73 was found.

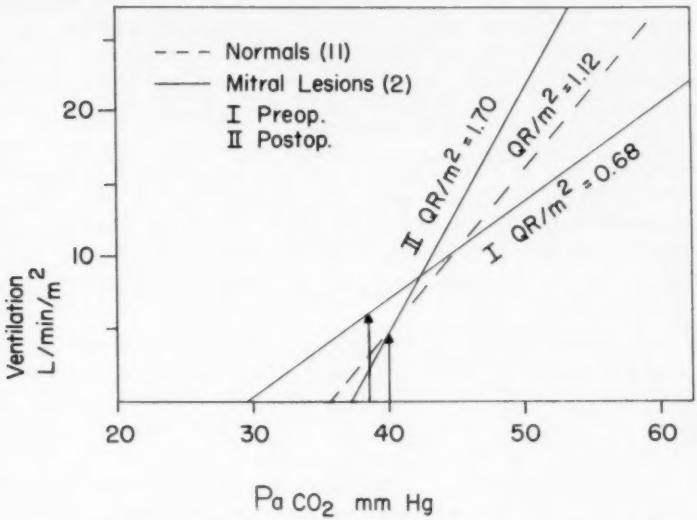

Fig. 2.—Straight lines showing mean relationship between ventilation and Pa_{CO_2} pre- and postoperatively in two patients with long-standing mitral stenosis who underwent successful commissurotomy. Arrows indicate levels of resting ventilation.

DISCUSSION

The most extensive previous studies of the relationship of ventilation to the carbon dioxide stimulus have been made in patients with diffuse obstructive emphysema, using a number of methods of artificial carbon dioxide accumulation. The decreased ventilatory response usually found in these individuals has been ascribed to (a) central depression of the respiratory center as a result of chronic hypercapnia,[1] or (b) mechanical limitation of the bellows.[11] The problem is complicated by the fact that the degree of activation of the respiratory center can be estimated only indirectly through its effects on the respiratory apparatus, which indeed is grossly altered in pulmonary emphysema. Although direct respiratory center depression in pulmonary emphysema has been seriously questioned,[11] observations in varying situations lend support to the existence of a true central alteration. A decrease in respiratory responsiveness, similar to that seen in pulmonary emphysema, has been found in normal individuals after prolonged exposure to a carbon dioxide enriched atmosphere, without change in the mechanics of breathing.[12] Conversely, ventilatory response to carbon dioxide has been returned toward

normal in a case of pulmonary emphysema by the prolonged use of a mechanical respirator which effectively lowered a chronically elevated Pa_{CO_2}.[13] In addition, two cases are on record[14, 15] of primary damage to the respiratory center, resulting in severe alveolar hypoventilation, hypoxia, and marked hypercapnia like that seen in advanced emphysema, in spite of no detectable abnormalities in the lungs, bellows, or pulmonary circulation. The outstanding defect in these individuals was almost total abolition of response to the CO_2 stimulus. Finally, regulatory changes taking place under exercise appear to change respiratory responsiveness,[16] as does administration of various drugs, such as morphine,[8, 17] salicylates,[18] and aminophylline,[19, 20] in both normal and emphysematous subjects.

Ventilatory response to carbon dioxide has also been studied in cyanotic heart disease and chronic metabolic acidosis and alkalosis.[1] In these 3 conditions, only chronic metabolic alkalosis with carbon dioxide accumulation was associated with diminished responsiveness of the respiratory centers. This again stresses the role of increased Pa_{CO_2} in reducing the responsiveness of the center.

On the other hand, altered mechanics of breathing undoubtedly play a role in modifying the effect of the CO_2 stimulus on the respiratory apparatus. Reports are available showing decreased ventilatory response to CO_2 in emphysematous patients with normal Pa_{CO_2}.[2, 5, 18] In addition, patients with emphysema have demonstrated a decreased ventilatory response to CO_2 in relation to their total O_2 uptake.[26] These studies thus lend support to the view that impaired mechanics of breathing may be a primary cause of reduced responsiveness to carbon dioxide in some cases.

In the present group of cardiac patients, a consistent hyperventilation at rest was found to be associated with two obviously interrelated types of altered response to CO_2. In the majority of cases, increased ventilation was accomplished by a lowering of the central threshold to the CO_2 stimulus (parallel displacement of the curve to the left). In only two individuals (I. M. and I. B.), both of whom were in the "cyanotic" congenital group, was there an abnormally increased responsiveness (QR) to a changing Pa_{CO_2} (increased slope of the stimulus-response curve) as described by Julich in the majority of his cardiac patients.[4] The difference in our results from those he reported is probably explained by the selection of subjects, since Julich's group was comprised of patients with primary myocardial disease as well as with valvular lesions.

The major finding in the present investigation was the significant depression of responsiveness to the CO_2 stimulus (QR) in the 7 patients with long-standing mitral disease. A complete reversal of the abnormal response was found postoperatively in two patients in this group who were studied after successful surgery, whereas no significant change occurred in the one patient in whom the operation was unsuccessful because of mitral insufficiency. Since postoperative resting Pa_{CO_2} and pH in the two successfully operated

patients did not vary significantly from their preoperative values, the restoration of the normal CO_2 sensitivity suggests that improved mechanics of breathing may be responsible for the change. Studies of ventilatory function in mitral disease have shown that measurements may be normal in the early phase, but that progressive reduction in maximum breathing capacity, and total and timed vital capacity, occurs after longer duration.[21] In addition, Marshall and co-workers[22] have demonstrated by direct means a gross impairment of the mechanics of breathing in long-standing mitral stenosis. Since pulmonary hypertension, increased pulmonary blood volume, and circulatory failure, rather than permanent pathologic abnormalities, appear to be the principal factors in the reduced compliance,[23] reversibility of the process might be anticipated following successful mitral valve surgery.

In contrast to the group with long-standing mitral disease, pre- and postoperative studies in one patient (E. B.) with mitral stenosis of relatively short duration showed a decrease in the central responsiveness to CO_2 after successful valvulotomy. Although no conclusions can be drawn from the data in one patient, they suggest the possibility that one ventilatory adaptation in cardiac patients is an increased responsiveness to the CO_2 stimulus as demonstrated by Julich,[4] whereas decreased responsiveness, as seen in our 7 patients with long-standing mitral disease, may occur in conjunction with the development of secondary pulmonary changes. Severe hypoventilation with consequent CO_2 retention might then be expected with more advanced pulmonary involvement. Although no patients of this type have been studied, clinical observations suggest that this course of events does occur in terminal mitral disease.

A somewhat similar situation may exist in certain primary hypoventilation states. If impairment of the mechanics of breathing can of itself reduce the respiratory response to CO_2, any increased demand for respiratory work may be expected to lead to pulmonary decompensation in persons whose ventilatory function is already border line. Recently, development of severe pulmonary insufficiency has been observed in patients with kyphoscoliosis and gross obesity coincident with the onset of congestive heart failure, with restoration of compensation as cardiac failure improves.[24, 25] Physiologic studies in these individuals suggest not only an abnormal central response to the CO_2 stimulus, but a reversion toward normal as the mechanical impairment of ventilation is improved.

SUMMARY

1. Ventilatory response to an increasing arterial carbon dioxide tension was studied in 11 normal individuals and 20 cardiac patients, using a rebreathing method for gradual increase of CO_2 in the inspired air.

2. A linear relationship between ventilation and arterial CO_2 tension was confirmed in both the normal individuals and cardiac patients.

3. A diminution of the respiratory "threshold" for CO_2, with consequent increase in resting ventilation was found in all cardiac patients.

4. Respiratory "responsiveness" to CO_2 was significantly diminished in the group of 7 patients with long-standing mitral stenosis. Studies after successful commissurotomy in two individuals suggested that these changes were reversible.

5. Impaired mechanics of breathing may be responsible for the diminished responsiveness to CO_2 in individuals with long-standing mitral stenosis, as appears to be the case in some patients with primary hypoventilation states.

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A HEMOLYTIC REACTION DUE TO THE TRANSFUSION OF A_x BLOOD

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A WEAK subgroup of the human blood group A, variously called A_x (A_4, A_5, A_z, or A_0), has been known since 1935.[1] Race[2] has suggested that the original notation, A_x, be used until the exact definitions of this rare blood are assigned. He has defined cells of this subgroup as giving the following agglutination reactions: "Negative or weak with Anti-A sera, negative with Anti-B, but positive with most Group O Sera (Anti-A plus Anti-B)."[2]

These bloods have been considered a potential danger in transfusion since they will be classified as group O both on the basis of direct grouping with known anti-A sera (made from immunized group B donors), and indirect (serum) grouping with known A_1 cells (since anti-A_1 may be present).

A_x antigens were found to produce anti-A antibodies when injected in small amounts into rabbits[3] and into group O human recipients.[4] The incidence of reactions resulting from such transfusions would depend on the incidence of the A_x factor. This is unknown. Some reactions of this type must occur, but because of their mildness go unnoticed. We are aware of only one report which may describe ill effects of inadvertent A_x transfusion to a group O recipient.[5] Blood from one donor, thought to be of group O, caused two fatal transfusion reactions in group O recipients. This took place in 1933 and 1934, before the A_x factor was described. The donor was later proved, with antiserum of higher titer, to be of group A.

Since this latter serum was probably the anti-A from a group B donor, it is likely that the dangerous donor was an A_2 or A_3 rather than an A_x. We wish to describe a hemolytic reaction caused by the inadvertent transfusion of proved A_x blood to a group O recipient.

CASE HISTORY

The patient was a 36-year-old white woman, with a history of acute leukemia of 1 year's duration. On April 14, 1958, she was admitted for the first time to the Washington Hospital Center. Treatment at other hospitals had included 28 whole blood transfusions, all of which were received without any known untoward reaction. The patient was treated with 6-mercaptopurine, prednisone, antibiotics, and butazolidin. Her blood group was determined to be O, Rh_0 positive. In the period between April 14 and April 30, 6 transfusions of 500 ml. each of Group O, Rh positive whole blood were given without untoward reactions.

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On May 1, two units of fresh whole blood were ordered for the treatment of profuse vaginal bleeding. Voluntary donors were contacted who were listed as Group O, Rh positive. The donors satisfied all standard criteria for donation and the blood was drawn with aseptic technique. Donor blood samples from pilot tubes were tested with 3 lots of anti-A and anti-B serum from two sources, using the slide technique recommended by the manufacturer. This triplicate direct grouping was done because indirect (serum) grouping was omitted. Each donor blood was determined to be group O, Rh positive. Cross matches between each of the donor bloods and the patient's were done using serum, high protein, and antiglobulin techniques as prescribed by the manufacturer of the reagent. These were read macroscopically and microscopically, and interpreted as compatible.

One hour after collection of the blood, the first transfusion was started and completed without incident. The second transfusion was then begun. Within 15 minutes the patient began to complain of substernal oppression, became nauseated and vomited. A sedative was administered by the resident physician, who elected to complete the transfusion. Substernal pain recurred and additional sedation was given. Transfusion of this unit was completed in 90 minutes at which time the patient complained of low back pain, and had a chill, followed by a temperature rise to 101° F. The urine which had been visually a clear yellow became reddish brown.

The patient's fever was controlled and after 3 hours she had no complaints.

Studies begun at this time showed no evidence of circulating incompatible cells (direct antiglobulin test negative), and the bilirubin content of her serum was 6.75 mg. per cent (direct 1.75, indirect 5.0). On the day after transfusion the patient's urine was again clear, and her serum was visually free of bile pigments. A base-line bilirubin obtained 10 days later was 0.23 mg. per cent.

The patient subsequently received transfusions on 7 occasions with group O, Rh positive blood, without any evidence of reaction. Twenty-nine days after the hemolytic reaction, the patient expired. An autopsy was performed and the primary diagnosis established as acute, aleukemic, granulocytic leukemia. The immediate cause of death was multiple visceral hemorrhages. Examination of the kidneys showed no gross or microscopic abnormalities. The liver and spleen demonstrated moderate hemosiderosis (a total of 43 transfusions had been given).

Posttransfusion Serologic Studies.—It was first suspected that this reaction was caused by passively transferred irregular antibodies in the plasma of the first donor, reacting with the cells of the second donor. This was disproved.

A further study of the blood of the second donor and the patient was then made as follows:

1. (a) *Cells versus selected grouping sera:* The donor red cells were tested (according to the manufacturer's directions) with a number of blood grouping sera which met the requirements for potency of the National Institutes of Health. The results of this testing are given in Table I. The cells were agglutinated by only one of 13 anti-A sera but by all 5 anti-AB sera (group O). The cells were not agglutinated by 4 anti-B sera.

TABLE I. DIRECT GROUPING OF DONOR BLOOD

	SELECTED GROUPING SERA		UNSELECTED DONOR SERA	
	ANTI-A	ANTI-AB (GROUP O)	ANTI-A	ANTI-AB (GROUP O)
Number positive	1	5	0	22
Number negative	12	0	11	5

(b) *Cells versus unselected donor sera:* The donor red cells were then tested for their complete (saline) reaction against sera from group O and group B donors. The tests were incubated at 22° C. for 30 minutes. Negative reactions were then retested at 15° C., after eliminating those sera which had cold agglutinin reactivity at this temperature. The

results are also given in Table I. With these unselected sera there was reaction with the anti-AB of 22 group O sera, but none with the anti-A of 11 group B sera. On the basis of these tests this donor blood was classified as being of subgroup A_x.

2. *Isoagglutinins.*—The donor's serum agglutinated A_1 cells microscopically after 30 minutes' incubation at 22° C. This became macroscopic when the test was carried out at 15° and 4° C. The serum did not agglutinate A_2 cells at these temperatures, but strongly agglutinated group B cells at 22° C.

In the pretransfusion serum of the patient, the titer of anti-A_1 was 1,024 by both the saline and antiglobulin techniques. The titer against B cells was 2,048 by the saline technique. It was 8 by both techniques against the cells of the A_x donor. Ten days after transfusion, the anti-A_1 titer was unchanged and the anti-A_x titer increased from 8 to 32.

3. *Cross Matching.*—Although the blood from this donor would be classified as group O on the basis of direct grouping tests (commercial anti-A sera from B donors) as well as reverse grouping tests (agglutinates A_1 cells as well as B cells), a cross match test with group O blood would be expected to disclose an incompatibility, especially when such a severe reaction could take place in vivo. Therefore, cross matching tests were done using several accepted methods. The following reactions were obtained between the pretransfusion serum of the patient and the donor cells:

(a) *Saline tube test:* Two volumes of serum, plus one volume of 2 per cent cells in saline; incubated 30 minutes at 22° C.—positive.

(b) *Indirect antiglobulin test:* Two volumes of serum, plus one volume of 2 per cent cells in saline; incubated 30 minutes at 37° C.—positive (4 different antiglobulin sera).

(c) *Albumin tube test:* Two volumes serum, plus one volume of 2 per cent cells in saline, plus two volumes of 22 per cent albumin; incubated 30 minutes at 37°C.—negative.

As can be seen, there was a positive reaction in the saline and indirect antiglobulin tests, but a negative reaction in the albumin tube test. The negative result was unexpected and it was thought that a prozone phenomenon might have inhibited the reaction. Titration experiments disproved this. In a further effort to determine the cause of this unexpected result, the pretransfusion serum of the patient was reacted with the donor cells using the different proportions of donor cells and media indicated in Table II. These variations of cross matching tests appear to confirm that the presence of albumin inhibited agglutination.

TABLE II. CROSS MATCH TESTS

PATIENT'S SERUM	MIXTURES A_x CELLS	ALBUMIN	RESULTS AFTER INCUBATION AT 37° C. FOR: 0	15 MIN.	30 MIN.	60 MIN.
2 vol. undil.	1 vol. 2%/saline	2 vol. 22%	Neg.	Neg.	Neg.	Neg.
2 vol. undil.	1 vol. 2%/saline	2 vol. 30%				Neg.
2 vol. undil.	1 vol. 2%/plasma	2 vol. 30%	Neg.		Neg.	Neg.
2 vol. undil.	"Stick" to 5%	1 vol. 30%	Neg.		Neg.	Neg.
2 vol. undil.	1 vol. 5%/30% albumin		Neg.		Neg.	Neg.
2 vol. undil.	1 vol. 2%/saline	None	Neg.		(+)	(W)
2 vol. undil.	"Stick" to 5%	None	Neg.		2+	2+

Reactions graded according to Race and Sanger.[6]

DISCUSSION

The search for the cause of a hemolytic reaction following an emergency transfusion to a group O recipient disclosed that the blood of one of the donors belonged to the rare subgroup of A called A_x. The incidence of this blood is

probably low in the general donor population. The donor described here is the third one known to us in this metropolitan area of approximately 1.5 million. This may account for the lack of reports of hemolytic reactions due to this unusual ABO incompatibility.

In this case, the high anti-A isoagglutinin titer of the recipient may have been responsible for the severity of the hemolytic process. It is believed that most of the A_x cells were immediately destroyed, since direct antiglobulin tests on the posttransfusion samples from the patient were negative. We cannot explain why the widely used albumin cross matching technique apparently inhibited the reaction between group O serum and these A_x cells.

The adoption of a simple test to disclose the A_x antigen would avoid the hazard of this type of incompatible transfusion. The routine use of anti-AB (group O) serum to check on blood which appears to be of group O is a practical test that is simple enough to be used in emergency circumstances.

SUMMARY

A nonfatal hemolytic transfusion reaction due to transfusion of blood of the subgroup A_x to a group O patient is reported. The red cells of the donor did not react with anti-A grouping sera nor with the patient's serum in the albumin (high protein) cross match but caused a severe clinical response.

We wish to thank Dr. J. Rheingold and Dr. E. Adelson for their permission to publish the clinical details in this case.

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THE RELATIONSHIP BETWEEN PLASMA PROTEINS AND RED CELL SEDIMENTATION IN A PROMETHAZINE SYSTEM

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IT IS a classic observation that human red cells in freshly drawn citrated whole blood sediment promptly, and that this process is usually fairly complete in 1 to 2 hours. During storage at +5° C., sedimentation behavior changes. At the end of about 4 days' storage, there is an increase in the time required for completion of sedimentation. Eventually, after approximately 7 days of storage, sedimentation does not occur.[1]

Variations in erythrocyte sedimentation rate must necessarily be related either to the properties of the medium in which the cells are being studied, i.e., composition, temperature, density, etc., or to properties of the red cells themselves, i.e., their number, shape, tendency to aggregation, surface tension, charge, etc., or to combined effects of these.

A special case of altered sedimentation behavior following change in the composition of the suspending medium was first described by Halpern, Dreyfus, and Bourdon[2] in studies of stored human blood. They noted that by adding small quantities of phenothiazine derivatives to the blood samples at the time of collection they obtained normal or near-normal sedimentation after storage periods of as much as 3 weeks. These observations were extended with one such phenothiazine derivative (promethazine hydrochloride) by Chaplin and associates.[3] They found that in spite of demonstrated stability of the compound in stored blood, they were unable to show an association of the improved in vitro characteristics with an increased in vivo survival of red cells which had been stored with autoclaved promethazine and then transfused.

It occurred to us that the 4-day period during which citrated whole blood samples maintain the relatively prompt sedimentation characteristic of fresh specimens, might correspond to the period during which the fibrinogen content remains at a high level.[4] Such an association would be in keeping with the observation[5-8] that fibrinogen level is the chief determinant of variations in sedimentation rate, although this has not always been uniformly demonstrated in in vitro studies.[9] The experiments to be reported were intended to explore directly the relationship of the sedimentation properties of whole blood to the plasma proteins, particularly fibrinogen, using in vitro samples subjected to varying periods of storage and to the presence of the phenothiazine derivative, promethazine hydrochloride.

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METHODS AND MATERIALS

Blood.—Whole blood was drawn from normal human donors by gravity under aseptic conditions into acid citrate dextrose solution (Formula A, USP) and was immediately mixed and divided into equal parts for study. Samples for parallel studies were all obtained from single venesections of single donors in order to control factors due to idiosyncrasies of the donor's red cell population and plasma. The donor was not taking promethazine or other antihistamine medication at the time of venesection. The blood was stored in glass containers at +5° C. Samples were not mixed during storage.

Promethazine Hydrochloride.—The compound promethazine hydrochloride, 10-(2-dimethylaminopropyl)phenothiazine hydrochloride (Phenergan hydrochloride),[10] was kindly supplied by Wyeth Laboratories, Inc. (Lot No. L1621b-1), prepared as a solution of 10 mg. per milliliter. It was sterilized by Seitz filtration and added to the experimental samples of whole blood to obtain a final dilution of 1:10,000.

At selected intervals during storage of the preparations, paired determinations were made on the experimental samples which contained promethazine and the control samples which did not.

The following determinations were made: sedimentation rate and hematocrit; fibrinogen level, determined in most cases by two methods; fibrinogen activity, as measured by thrombin time determinations; and plasma protein electrophoresis.

1. Sedimentation rates were measured using Wintrobe tubes in a leveling rack. Readings were taken over a 3-hour period. Hematocrit values were obtained at the end of each period by centrifuging the Wintrobe tubes at 1,800 g for 30 minutes.

2. Plasma protein electrophoresis curves were obtained using the method of Jencks and associates,[11] with barbiturate buffer at pH 8.6. The strips were scanned using the Spinco Analytrol. Figures are given for each peak as per cent of total protein.

3. Fibrinogen determinations were made in most cases by micro-Kjeldahl determination and also by the method of Christensen,[12] namely, trypsin digestion of the fibrin clot and subsequent spectrophotometric determination of amino acid content at 280 mμ.

4. Thrombin time determinations were carried out by preparing commercial thrombin solution in an initial dilution of 10 units per 0.05 ml. which was calibrated to give the end point, a discernible clot, in about 20 seconds with the addition of 0.5 ml. of normal plasma.

Most of the determinations described were made after 2, 4, 10, and 17 days of storage of the blood. These days were chosen for the following reasons: on the second day, both experimental and control preparations would be expected from the previous work, confirmed in our preliminary experiments, to show the sedimentation behavior of fresh blood; on the fourth day, the control samples would be expected to show transition values, and the experimental samples to be relatively unchanged; on the tenth day, the maximum differences in sedimenting behavior between the experimental and control samples would be anticipated; on the seventeenth day, the experimental sample might begin to show diminished sedimentation rate.

RESULTS

A. Protein Studies.—

1. The results of serum protein analysis by the chromatographic method are presented in Table I. There is no evidence of significant alteration in any of the plasma protein levels studied, either as a result of storage per se, or as a result of adding promethazine.

2. The fibrinogen determinations done by micro-Kjeldahl analysis were made on certain specimens only, as shown in Table II. There is no evidence

of significant change in the fibrinogen concentration over the 17-day period of observation. Three additional sets of paired observations submitted to analysis by the spectrophotometric method likewise gave no indication of change in fibrinogen level associated with either duration of storage or addition of promethazine.

TABLE I. PLASMA PROTEIN ON STORAGE (ELECTROPHORESIS, PER CENT TOTAL PROTEIN)

EXPERIMENT		ALBUMIN	ALPHA-1	ALPHA-2	BETA	GAMMA
Day 2	Control	52	9	8	13	18
	Promethazine	64	3	8	8	17
Day 4	Control	65	4	6	9	16
	Promethazine	61	4	9	10	16
Day 10	Control	64	4	8	9	15
	Promethazine	60	5	7	12	16
Day 17	Control	61	5	8	7	19
	Promethazine	66	4	8	9	13

TABLE II. PLASMA PROTEIN ON STORAGE (MICRO-KJELDAHL, MILLIGRAMS PER CENT)

DAYS OF STORAGE	CONTROL	PROMETHAZINE
2	171	182
10	179	180
17	—	187

TABLE III. THROMBIN TIME

EXPERIMENT	THROMBIN TIME (SECONDS)*			
Day	2	4	10	17
Control	19.6	19.3	17.0	17.0
Promethazine	19.1	18.5	18.3	15.0

*Each figure represents the average of 6 individual determinations.

3. The data giving fibrinogen activity in terms of thrombin time are presented in Table III. There is no evidence that the presence of promethazine altered thrombin time in a significant manner.

B. Cell Surface Studies.—Because of the definite effect of adding promethazine on the sedimentation rate, without evidence of associated alteration in either amount or activity of fibrinogen or other plasma proteins, we investigated changes in the red cell surface as a possible basis for the effect.

It was demonstrated, first, that promethazine need not be present during the storage period to obtain its accelerating effect. For control preparations without promethazine made on the seventeenth day, the sedimentation rate was 3 mm. per hour. For experimental preparations made with promethazine in the usual manner by adding the compound before storage, the sedimentation rate at the end of one hour was 28 mm. For preparations to which promethazine was added just prior to reading, the sedimentation rate was 24 mm. per hour. In other words, prompt sedimentation like that of the *fresh* red cells was obtained with *aged* red cells when promethazine was added to the sample just before the determination was made. The inference is that the sedimentation storage lesion is readily reversible by promethazine.

It is known that washing red cells (with isotonic saline) tends to produce a sphering effect. It was found in our experiments that washing the cells to which promethazine had been added, with removal of the compound from the preparation at the time of the sedimentation rate, produced sedimentation determination times similar to those of control preparations without promethazine. The indication is that the promethazine combination must be a mere coating of the surface or must be very loosely adsorbed by the surface.

To study the nature of the surface changes, preparations were made which varied with respect to length of storage, presence or absence of promethazine, and saline washing of cells, using a new series of donors. These preparations were all adjusted to approximately equal hematocrits of 20 ± 1.

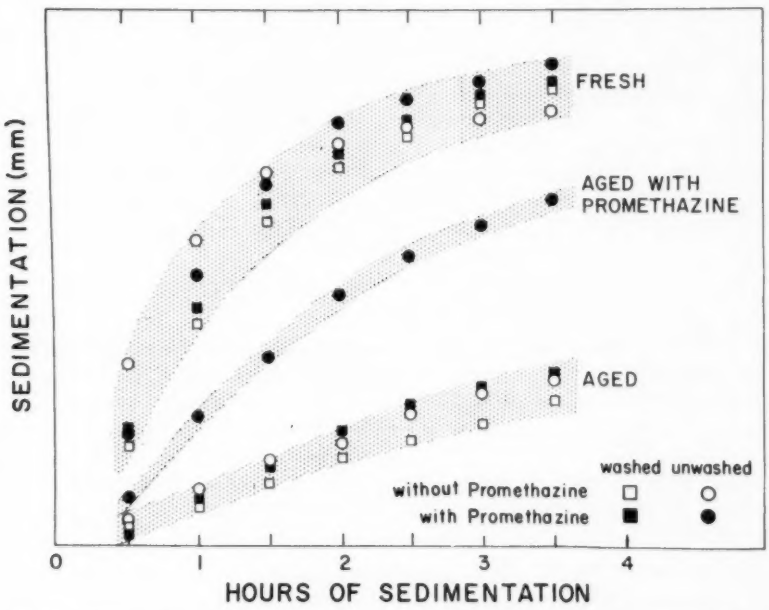

Fig. 1.—Sedimentation rates in R.B.C. systems studied under varying conditions of cell age, washing, and promethazine content.

The results are shown in Fig. 1. It can be seen that at this hematocrit the fall varied from 20 to 60 mm. at the end of the 3½ hour observation period. All the fresh cell preparations sedimented rapidly, more so than any of the ones made with aged cells. The effect of washing and of adding promethazine on the fresh cells appeared negligible. The preparations of aged cells all showed more rapid sedimentation when promethazine was present than when it was not. (This was true whether promethazine was added at the time of venesection and present during the entire storage period or added just

prior to reading the sedimentation rate.) In general, washed preparations appeared to sediment less rapidly than comparable unwashed preparations. It seems possible to state:

1. The fresh cell preparations showed consistently more rapid sedimentation than the aged, regardless of status with respect to promethazine or washing.

2. The addition of promethazine to aged cell preparations tended to produce values approaching those for fresh preparations.

3. Washing the cells tended to delay sedimentation.

4. There is an interaction of these effects, so that those which tend to accentuate sedimentation rate can be affected by those tending to delay it, i.e., the accelerating effect of promethazine is counteracted in part by the delaying effect of washing.

DISCUSSION

The data presented have reconfirmed the observation of others that promethazine tends to maintain normal sedimentation behavior of whole blood samples during storage. Therefore, this represents a special case of alteration in the sedimentation system. In addition, we found that this material need not be present during the storage period but is active if added to old cells just before testing (see findings of Schales[12] for osmotic fragility data in a comparable system).

Previous attempts to find the basis for variability in sedimentation phenomena, particularly as related to promethazine systems, fall into 3 major groups. First is the possibility that plasma constituents other than those we studied, especially proteins present in small but enzymatically active amounts, or nonprotein elements, would be critical. Several investigators[9, 13] have made suggestions to this effect, but no such factor has been demonstrated. A second type of explanation localizes the critical changes affecting sedimentation rate at the cell surface. The features considered by various authors have included changes in the charge density,[13, 14] although such changes have not been studied in the promethazine system; permeability, as detected by ion exchange studies, particularly K^{+},[1, 12, 15]; fragility[12, 15-17]; oxygen capacity[18]; and shape (sphering).[3, 19] No differences in permeability have been demonstrated as a result of adding promethazine to the system, but it has been consistently shown that promethazine retards the increase in osmotic fragility characteristics of stored erythrocytes. Grieg and Gibbons[20] have shown that promethazine prevents loss of cholinesterase from the red cell surface during storage, and that this may be the crucial factor or one of the crucial factors accounting for improved red cell preservation. Finally, it has been thought that changes in the R.B.C. stroma[16] or over-all R.B.C. metabolism[15, 18] were implicated; electron microscopy[19] seems to show that rearrangement of stromal elements may occur in the presence of "antisphering" compounds, including phenothiazine derivatives. The significance of the microscopic changes is not clear at present.

The results of our studies support the view that alterations in the plasma protein, particularly fibrinogen, are not responsible for the promethazine effect. Taken in conjunction with several studies showing that promethazine retards the increase of osmotic fragility, and the results of metabolic studies thus far performed, our results seem to point to the red cell surface as the site of action of the compound in altering erythrocyte sedimentation rate.

SUMMARY

It is known that stored blood samples show increasingly slow erythrocyte sedimentation rates, and that certain antihistamine compounds, such as promethazine hydrochloride, delay the appearance of this phenomenon. The possible relationship of these features of sedimentation behavior to alterations in plasma protein levels was studied, but no significant relationship was demonstrated.

Additional in vitro studies directed toward identifying the mode of action of promethazine implicated the red cell surface as the probable site of action. The relationship of this work to other studies is discussed.

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INTESTINAL LOSS AND REABSORPTION OF IRON IN HOOKWORM INFECTION

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WITH THE TECHNICAL ASSISTANCE OF ABRAHAM LEVY

A CONSIDERABLE amount of blood may be lost into the gastrointestinal tract by patients infected with hookworm.[1, 2] It appeared interesting to determine whether the iron from that blood was totally lost in the feces, or whether part of it was reabsorbed. A method has been described to measure the amount of iron lost into the intestine and later reabsorbed.[3] Briefly, the circulating erythrocytes are marked with both radioactive chromium (Cr^{51})* and radioactive iron (Fe^{59}).* Radioactivity from either isotope is measured with the help of a pulse height analyzer in blood and in feces.[4]

It has been shown that Cr^{51} is not significantly excreted in the feces when it is incorporated into circulating erythrocytes and that only small amounts of Cr^{51} are reabsorbed from the intestinal lumen.[1] Fecal radioactivity from Cr^{51} may then be taken to indicate the amount of blood which is being lost into the intestine. From the blood hemoglobin concentration, intestinal iron loss can then be calculated. Radioactivity from Fe^{59} indicates the actual amount of iron lost into the feces. By subtraction, the amount of iron reabsorbed may be calculated.

The present paper describes the results obtained with the above method in 14 patients infected with hookworm.

METHODS

The methods for fecal and blood collection, sample preparation, tagging with isotopes, and measurement of radioactivity have been previously described.[1, 3] Hemoglobin was determined by the cyanomethemoglobin method.[5] Serum iron was determined by the method of Peters and associates.[6] All patients were hospitalized in the Hospital Vargas and were studied for at least 3 periods of 4 days each. All of them, with the exception of R. A. entered the hospital because of anemia. R. A. had rheumatoid arthritis. Ova were counted at least 3 times in each patient by the method of Caldwell.[7]

RESULTS AND DISCUSSION

The results may be seen in Table I and in Fig. 1.

It appears clear that iron from hemoglobin is in large part reabsorbed after it is lost into the intestine through the action of the hookworm (Table I

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*From Abbott Laboratories, Oak Ridge, Tenn.

TABLE I. INTESTINAL LOSS AND REABSORPTION OF IRON IN HOOKWORM INFECTION

PATIENT, AGE, AND SEX	BLOOD HEMOGLOBIN (GM./100 ML.)	PLASMA IRON (μG/100 ML.)	OVA IN FECES (MILLION/DAY)	NUMBER OF WORMS RECOVERED	PER CENT ANCYLOSTOMA DUODENALE	PER CENT NECATOR AMERICANUS	INTESTINAL BLOOD LOSS (ML./DAY)	IRON LOSS (MG./DAY)		REABSORPTION	
								INTESTINAL	FECAL	MG./DAY	PER CENT
E. J. 27 M	3.4	52	11.39	3,861	0	100	100.2	21.34	16.24	5.10	23.9
P. G. 60 M	7.2	60	6.54	382	0	100	82.8	17.93	9.75	8.18	45.6
A. Z. 16 M	6.4	56	11.18	1,483	4.0	96.0	107.3	17.51	9.15	8.36	47.7
F. A. 70 M	6.5	-	2.14	-	-	-	32.3	13.58	3.21	10.37	76.4
E. C. 48 M	6.3	30	4.00	491	0	100	68.3	9.68	5.87	3.81	39.4
J. D. 30 M	3.2	21	5.99	239	0	100	86.9	9.21	4.93	4.28	46.5
C. C. 15 F	5.9	15	4.51	1,355	0	100	53.6	8.40	3.18	5.22	62.1
D. M. 13 M	3.1	82	3.26	740	0	100	44.4	7.19	3.52	3.67	51.0
C. O. 60 M	11.0	-	0.58	102	3.6	96.4	20.0	6.54	2.82	3.72	56.9
P. G. 33 M	4.5	43	5.36	1,493	0	100	47.6	6.27	3.66	2.61	41.6
F. R. 22 M	9.4	28	3.88	925	0	100	27.0	5.86	3.27	2.59	44.2
L. N. 22 M	4.5	25	2.43	2,240	0	100	17.3	4.28	2.82	1.46	34.1
R. A. 23 M	14.0	20	0.66	206	0	100	8.9	3.34	2.90	0.44	13.1
B. C. 30 M	4.8	32	1.24	284	0	100	18.8	2.74	1.80	0.94	34.2

and Fig. 1). The per cent absorption, which varies between 13.1 and 76.4 (average 44.1) is in general considerably higher than that found by Callender, Mallet, and Smith.[8] These workers fed Fe^{59}-labeled rabbit hemoglobin to normal and anemic human subjects. With raw hemoglobin, they found an average of 10 per cent of the ingested Fe^{59} in the circulating erythrocytes in 11 normal subjects and 22 per cent in 11 anemic patients. With cooked hemoglobin, the average found in erythrocytes was 7 per cent in the normal and 12 per cent in the anemic. It must be pointed out that in the majority of these subjects, blood volume was not measured, but calculated on the basis of body weight.

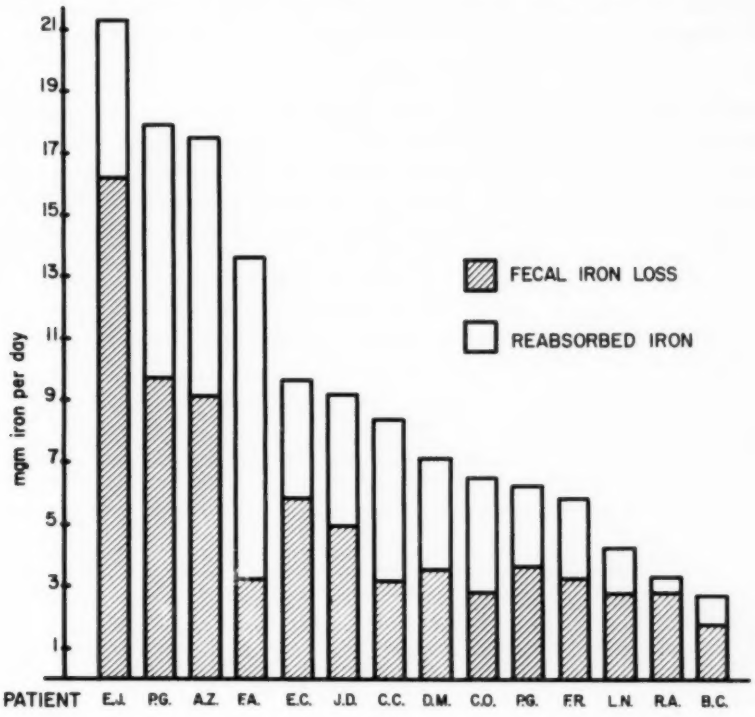

Fig. 1

Moore,[9] on the other hand, found in normal subjects less than 10 per cent iron absorption from eggs, chicken liver, spinach greens and lettuce, and dried yeast; Steinkamp, Dubach and Moore[10] found the absorption of various iron salts baked into bread to vary between 1 and 12 per cent in 28 normal subjects and between 26 to 38 per cent in the other 4, in whom there was reason to suspect that iron stores were suboptimal. In 3 frankly iron-deficient subjects, absorption was 45, 57, and 64 per cent, respectively. It may be, therefore, that the generally high reabsorption found in the present series was due to diminished iron stores, although no correlation was found between blood hemoglobin

values, serum iron concentration, and per cent reabsorption of iron. There was no direct or inverse correlation either between quantity of iron lost and per cent reabsorbed.

It is possible that hemoglobin may eventually be transformed as it passes through the hookworm's intestinal tract, so that its iron becomes available for intestinal absorption by the host. In view of the finding of Wells[11] and Nishi[12] that, in *Ancylostoma caninum*, the dog's red blood cells appear intact when they come out of the hookworm's anus, this supposition appears unlikely. A study of noninfected anemic patients with slowly bleeding intestinal lesions and a comparison of these patients with those in the present series might further settle this point.

SUMMARY

The circulating erythrocytes of 14 patients with hookworm infection were marked with both radioactive chromium (Cr^{51}) and radioactive iron (Fe^{59}). Radioactivity from either isotope was measured in blood and feces with a pulse height analyzer. Radioactivity from Cr^{51} was taken to indicate how much blood and iron was being lost through the hookworms, and radioactivity from Fe^{59} indicated the amount of iron actually lost through the feces, while the difference between the two values gave the quantity of iron reabsorbed. Intestinal iron loss varied between 2.74 and 21.34 mg. per day, while fecal loss ranged from 1.80 to 16.24 mg. per day. On the average, 44.1 per cent of the intestinal iron lost was reabsorbed, with a range of 13.1 to 76.4.

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THE EFFECT OF HYPAQUE AND TELEPAQUE ON THYROID UPTAKE OF I^{131} AND PLASMA PROTEIN-BOUND IODINE

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WITH the wide usage of the thyroid radioiodine uptake and the protein-bound iodine (PBI) tests in evaluation of thyroid function, it is important to define some of the limitations and sources of error in these procedures. One common problem is the artifactual elevation of the PBI and lowering of the thyroid uptake which follows administration of organic iodine compounds in excretory urography and oral cholecystography. A number of studies of the effect of various cholecystographic and urographic media on the I^{131} uptake by the thyroid and on the PBI have resulted in conflicting information. The present study is concerned with the duration of influence of Hypaque (diatrizoate sodium) and Telepaque (iopanoic acid) in the euthyroid subject.

Dobyns[1] has claimed that following pyelography the radioiodine test is probably not reliable for 3 or 4 weeks. In euthyroid patients, Beierwaltes and associates[2] found the PBI still elevated 36 days following Urokon and 39 days after Telepaque. After Urokon the I^{131} uptake returned to normal in 31 days. Fields and Seed[3] state that the effects of the gall bladder and kidney visualization media last for many weeks. Drummy[4] reported depression of the 24-hour thyroid uptake of radioiodine to less than 15 per cent (normal range: 15 to 50 per cent) for at least one week following urography in 7 of 8 euthyroid patients; in 1 hyperthyroid patient, the level was above 15 per cent within 3 days.

It is generally agreed that, due to reabsorption from the bowel, the oral contrast media used for gall bladder visualization exert a more prolonged effect than the intravenous urographic media and that for either class of compounds the thyrotoxic patient escapes more rapidly than the euthyroid individual. Slingerland[5] found in hyperthyroid patients high or high normal thyroid uptake within one week after oral cholecystography with Priodax, whereas euthyroid patients had depressed thyroid uptakes usually up to 4 weeks. In the patients reported by Slingerland, the time required for the PBI to return to normal levels was longer than the time for thyroid uptake levels to become normal. Nine euthyroid patients studied 2 months or less following Priodax had PBI values greater than 8 μg per 100 ml., whereas only one of 12 patients studied between two and 12 months had a PBI value greater than

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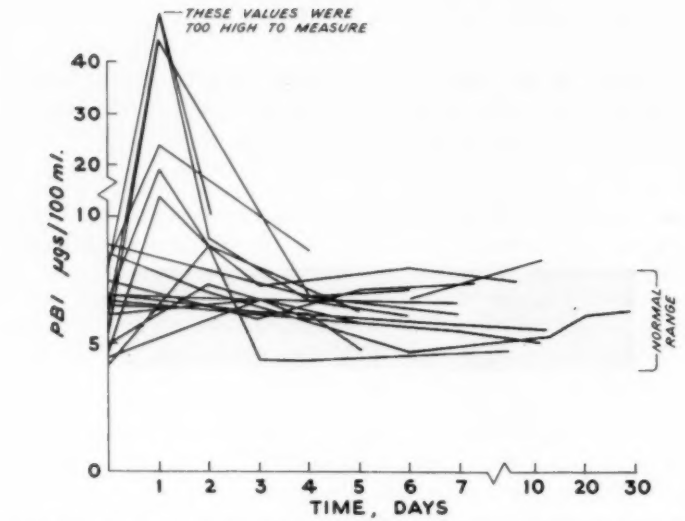

Fig. 1.—Effect of 30 ml. of 50 per cent intravenous Hypaque on the serum protein-bound iodine level.

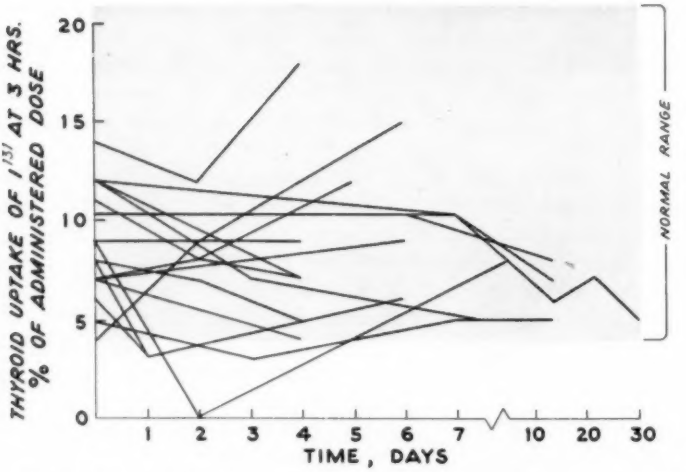

Fig. 2.—Effect of 30 ml. of 50 per cent intravenous Hypaque on the 3-hour uptake of I^{131} by the thyroid. Data for 5- and 24-hour uptakes show effect similar to that seen at 3 hours.

8 μg per 100 ml. Freedberg[6] reported one thyrotoxic patient whose I^{131} uptake 5 weeks following cholecystography was in the thyrotoxic range. Drummy[4] found the 24-hour radioiodine uptake in euthyroid patients after oral Priodax was usually below 15 per cent for at least 6 months, although in a few of his patients, the uptake had returned to low normal values within two months.

On the other hand, Newman and Cupp[7] found that each of the 6 euthyroid patients studied had normal 24-hour uptake values within 30 days after receiving 3 Gm. of Priodax. Clark and Shipley[8] studied the 24-hour I^{131} uptake following the oral administration of 3 Gm. of Telepaque in 74 euthyroid subjects and found that the average value was back to 60 per cent of the average pre-Telepaque level in 30 days and to 90 per cent within 60 days.

METHOD

A total of 25 euthyroid patients was studied, 19 following Hypaque and 6 following Telepaque. None had any indication of thyroid abnormality and there was no history of interfering medicinal or chemical agents. Tracer doses of I^{131} varying from 10 to 50 μc were given by mouth to the fasting patient, and thyroid uptake measurements were made at intervals of 1, 3, 5, and 24 hours following the test dose; not all patients had measurements at each of these times. The effect of these media on the 3-, 5-, and 24-hour measurements was the same. PBI specimens were also obtained fasting. Base-line PBI and thyroid uptake studies were made before administration of Hypaque or Telepaque in all except 3 cases. The normal range for PBI in this laboratory is 4 to 8 μg per 100 ml. The normal range for I^{131} uptake is about 4 to 24 per cent at 3 hours after the test dose, 5 to 30 per cent at 5 hours, and 15 to 45 per cent at 24 hours.

Follow-up studies were performed at varying intervals following administration of the contrast media. After the base-line study, all data for radioiodine content in the thyroid were corrected for the amount of I^{131} present in the thyroid just before each new test dose. To further minimize the error resulting from the thyroid residual of I^{131}, successive test doses were progressively larger. The urogram patients received 30 ml. of 50 per cent Hypaque by vein; each patient in the Telepaque group received 3 Gm. of the material orally.

Allen[9] and Werner[10] have noted only slight variation in serial thyroid uptake studies performed on healthy euthyroid adults. In our experience, using correction for residual I^{131} and graded tracer doses of I^{131}, this error has been small; repeat determinations on the same patient gave comparable results. According to Danowski and associates[11] the PBI level also is relatively constant over a period of weeks.

RESULTS

Hypaque.—Each of 7 patients in whom a PBI specimen was obtained the first day following urography showed a pronounced elevation varying from 13.7 μg per 100 ml. to "too high to measure" (Fig. 1). Seven patients had specimens taken the second day; 5 of these showed slight elevation whereas the other 2 were within the range of normal. All 6 patients having specimens on the third post-Hypaque day showed normal values. With one exception, which occurred after two weeks and was probably a laboratory error, all specimens drawn after the fourth day were in the range of normal.

Five of 6 patients who had thyroid I^{131} uptake measurements the first post-Hypaque day showed depression of the curve (data for patients with measurements made at 3 hours are given in Fig. 2). The effect on the uptake curve

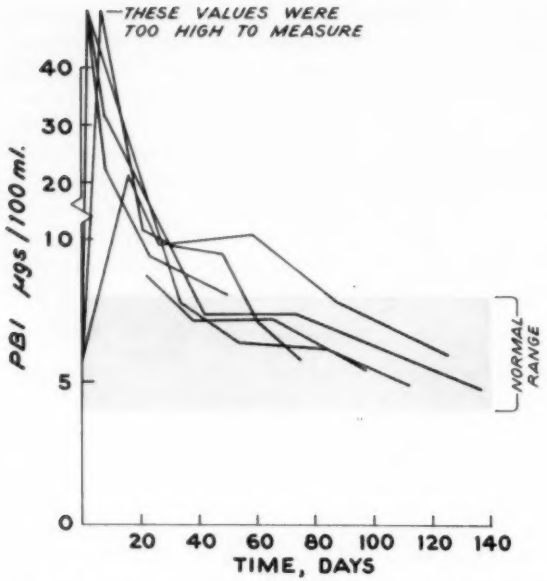

Fig. 3.—Effect of 3 Gm. of oral Telepaque on the serum protein-bound iodine level.

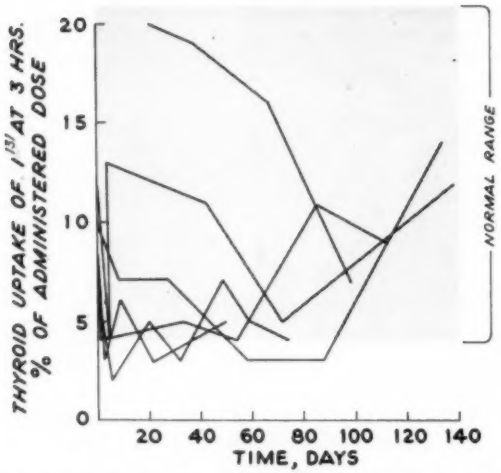

Fig. 4.—Effect of 3 Gm. of oral Telepaque on the 3-hour uptake of I^{131} by the thyroid. Data for 5- and 24-hour uptakes show effect similar to that seen at 3 hours.

was slower in reaching maximum and was less marked and less predictable than the effect on PBI. Nine patients had uptake measurements on the second day; 4 of these were depressed, and 5 had returned to normal. Seven of 8 patients having thyroid measurements on the fourth day showed normal results, and all thyroid uptake studies performed later than the fourth post-Hypaque day were found to be within normal limits. Thus, in the euthyroid patient given 30 ml. of 50 per cent Hypaque, PBI and I^{131} uptake values were within the normal range by 4 days.

Telepaque.—The studies on the patients receiving Telepaque were done at 3- to 4-week intervals so that the time of escape of the PBI from the spurious elevation is less sharply defined than in the Hypaque studies (Fig. 3). Excluding one patient whose last specimen 50 days after Telepaque showed borderline elevation, the average time for the PBI value to return to the normal range was 52 days. In the 4 patients studied after return of the PBI to within the normal range, the PBI continued to slowly decrease and usually reached the pre-Telepaque level only after an additional several weeks. At one extreme was a patient whose value returned to normal within 33 days whereas the longest escape time noted was 89 days.

The thyroid radioiodine uptake escape time showed greater variation (data for patients with measurements made at 3 hours are given in Fig. 4). As with Hypaque, the effect of Telepaque on the uptake curve tended to be slower in appearing and less predictable than the effect on the PBI. The shortest escape time was 7 days; the longest, 131 days. Excluding one patient whose uptake was depressed when last measured on the fiftieth day, the average was 58 days. As with the PBI, pre-Telepaque levels were sometimes not reached until several weeks after the values for uptake had returned to within the normal range.

SUMMARY

Nineteen euthyroid patients were studied before and after the intravenous administration of Hypaque and 6 before and after oral Telepaque to determine the duration of effect of these materials on the thyroid I^{131} uptake and on the serum precipitable iodine. After Hypaque, the previously depressed thyroid uptake returned to normal range in all patients by the fourth post-Hypaque day, and the elevation of the protein-bound iodine was no longer present after the fourth day. Following Telepaque, patients exhibited uptake measurements considered normal in an average of 58 days. The corresponding protein-bound iodine values became normal within an average of 52 days.

We are indebted to Miss Josephine Sommers for her technical and clerical assistance.

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THE RATE OF DISAPPEARANCE FROM PLASMA AND SUBSEQUENT DISTRIBUTION OF RADIOCADMIUM (Cd^{115m}) IN NORMAL DOGS

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INCIDENT to studies concerned with cardiac hypertrophy and dilatation, attention was directed to the development of a simple, reliable technique for inducing cardiomegaly in experimental animals. The reported occurrence of cardiac enlargement in chronic cadmium poisoning[1, 2] prompted investigation of the kinetics of cadmium metabolism. Although subsequent investigations have shown that cadmium probably is not a satisfactory agent to induce cardiomegaly, much information concerning the kinetics of cadmium metabolism was obtained and considered worthy of report. This is particularly true since cadmium seems to be a trace element of importance in certain enzyme activity,[3, 4] and has interesting aspects of tissue distribution. These experiments supplement important studies of Freiberg.[5-9].

METHODS AND MATERIALS

Cd^{115m} (half-life, 43 days) was obtained from Oak Ridge National Laboratories as cadmium nitrate in excess nitric acid which was neutralized with sodium hydroxide before use. Because of the high toxicity of cadmium and the low specific gravity of the Cd^{115m}, the radiation dosages employed were small (10 to 20 μc) and the counts lower than desired (350,000 and 450,000 c.p.m. per kilogram containing 0.241 to 0.360 mg. of cadmium nitrate per kilogram of body weight) to avoid intoxication from the carrier cadmium.

Four healthy mongrel dogs were placed in separate metabolic cages for several days prior to administration of the tracer and were maintained on the same diet (one pound of Pard* per day) which was continued throughout the experiment. Water was allowed as desired. After intravenous pentobarbital anesthesia (30 mg. per kilogram of body weight) an indwelling heparinized needle was placed in the femoral vein and Cd^{115m} was injected as rapidly as possible (one to two seconds) into another vein of the opposite leg. Initially, samples of venous blood were drawn for radiocadmium assay at 5-second intervals, then with decreasing frequency during 120 minutes.

Blood samples were collected with and without heparin as an anticoagulant. The heparinized sample of blood was centrifuged in Wintrobe hematocrit tubes and the supernatant plasma removed for counting. The buffy coat was removed and the residual erythrocyte blood cells were washed 3 times with human Ringer's solution before counting to determine the rate of entry of Cd^{115m} into the erythrocytes. The buffy coat collected from several samples counted separately, failed to reveal the presence of significant

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*Canned dog food manufactured by Armour & Company, Chicago, Ill.

amounts of radiocadmium. Finally, an aliquot of whole blood was laked by freezing and counted to determine the radioactivity of whole blood. Serum was obtained from routine centrifugation of the nonheparinized samples of whole blood for determination of the concentration of Cd^{115m} in serum.

The animals were sacrificed at intervals of 2, 24, and 48 (2 dogs) hours after the intravenous injection was made. In the interim, they were in metabolic cages where urine, feces, and cage washings were collected each morning and assayed for radiocadmium. Tissue specimens were obtained from the organs listed in Table II, were washed quickly with water, blotted dry, placed in weighing bottles, weighed to the nearest tenth of a milligram, and transferred to digestion flasks. Digestion was accomplished with concentrated nitric acid and heating; excess nitrates were driven off with formic acid according to the method of Sheppard and Martin.[10] The contents of the flasks were transferred to 5 or 10 ml. volumetric flasks and diluted to the volume of the flasks.

All counting was made with a thin mica end window Geiger-Müller counter, using methods previously described.[11] Dogs A and B were studied prior to refinement of the study of the tissues. Thus, the organ distribution presented should be considered to be qualitatively accurate for all dogs, but quantitatively accurate only for Dogs C and D.

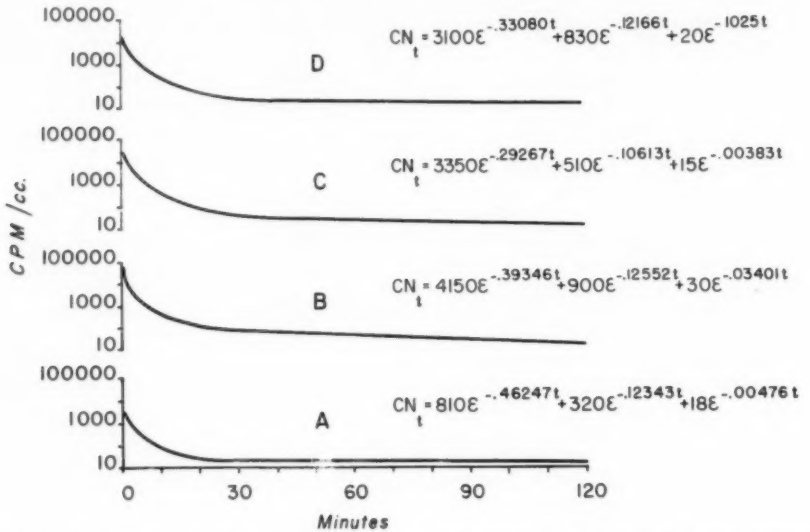

Fig. 1.—Time course of plasma concentration of radiocadmium in 4 dogs following intravenous injection.

RESULTS

The concentration-time course of Cd^{115m} in the plasma: The concentration-time course of Cd^{115m} in the plasma of 4 dogs is presented in Fig. 1. The configuration of the curves is similar in general appearance to those obtained for Na^{24}, Na^{22}, Cl^{36}, Hg^{203}, and Rb^{86}, but is quantitatively different.[12-16]

The distribution and rate of exchange of Cd^{115m} between the plasma and extra plasmal compartments (Table I) were calculated from the regression curves (Fig. 1) according to the method of Merrell and associates.[17, 18] The

concentration-time course curves and exchange rates were similar for the 4 dogs. The values for Dog A differed from those of the other 3 animals because of a slight perivascular infiltration of the tracer at the time of injection.

TABLE I. RATE OF EXCHANGE OF Cd^{115m} BETWEEN THE PLASMAL AND EXTRAPLASMAL COMPARTMENTS OF 4 DOGS

DOG	A	B	C	D
Proportion of total Cd^{115m} not in plasma	0.984	0.994	0.996	0.995
Proportion of total Cd^{115m} in plasma	0.016	0.006	0.004	0.005
Proportion of seral Cd^{115m} leaving the plasma per minute	0.360	0.344	0.267	0.286
Proportion of nonseral Cd^{115m} returning to the plasma per minute	0.0057	0.002	0.001	0.001
Proportion of total body Cd^{115m} moving in or out of the plasma per minute	0.0056	0.002	0.001	0.001
Proportion of total body Cd^{115m} moving in and out of the plasma per minute	0.0113	0.004	0.002	0.002

DISCUSSION

Of considerable interest is the almost complete disappearance of Cd^{115m} from the plasma during the second hour of the experiment, due to movement to the extraplasmal (primarily extravascular) compartments. This is consistent with temporary or fairly permanent fixation, possibly by chelation, as evidenced by the slow urinary and fecal excretion noted in this and other experiments. There was minimal return of Cd^{115m} to the plasma after it once left that compartment (Table I). Moreover, that portion of the isotope which entered the erythrocytes during the first hour of the experiment remained at essentially the same concentration in samples collected 24 and 48 hours after the injection. Further evidence for firm fixation of cadmium in tissues was obtained during the study of Dog A, in which slight perivascular infiltration occurred at the time of injection. Forty-eight hours later, specimens of muscle and vessel from the area of injection contained from 5,000 to almost 100,000 c.p.m. per gram of wet tissue, a high concentration in comparison to that for other tissues (Table II).

The affinity of sulfhydryl groups for cadmium is well established.[3, 19] However, the affinity of thiols in general for sulfhydryl poisons varies considerably. Thus, the problem of transport and in vivo fixation of cadmium must be complex, with many chemical systems involved. Hughes's explanation[20] of the behavior of mercury probably applies, at least in part, to cadmium, i.e., the principal reaction is with thiols which provide a mechanism of transport and fixation. Other chemical reactions may be concerned which may involve amino, hydroxy, imidazole, or carboxyl groups.[21]

Organ Distribution.—Table II lists the tissues examined for Cd^{115m} and the concentrations found. It should be noted that the data are qualitatively accurate in all animals, but quantitatively accurate only in Dogs C and D. The

TABLE II. TISSUE DISTRIBUTION OF Cd^{115m} IN C.P.M. PER GRAM OF WET TISSUE FOLLOWING INTRAVENOUS INJECTION OF TRACER. DOSAGE WAS ADJUSTED TO 425,000 C.P.M. PER KILOGRAM OF BODY WEIGHT. TIME OF COLLECTION OF TISSUES AFTER INJECTION OF TRACER IS SHOWN FOR DOGS A TO D

TISSUES	A (48 HR.)	B (48 HR.)	C (24 HR.)	D (2 HR.)
Adrenal	1,408	632	47	202
Bladder	28	241	175	0
Diaphragm	65	90	74	176
Endomyocardium outer third	754	837	239	692
Epimyocardium	128	454	478	318
Esophagus	167	72	126	23
Eyeball	0	25	25	53
Gall bladder	1,584	1,448	514	380
Large intestine	532	933	407	539
Left auricle	337	888	377	808
Left ventricle	1,188	440	405	630
Liver	5,672	8,687	10,403	8,892
Lung	219	241	174	395
Mediastinal node	1,878	1,385	445	519
Mid-myocardium	876	806	506	520
Ovary	*	*	225	*
Pancreas	984	1,403	721	4,600
Papillary muscle	*	*	180	315
Pericardium	59	39	135	173
Renal cortex	5,605	4,466	1,336	2,380
Renal medulla	1,024	3,748	648	0
Retina	*	*	469	80
Right auricle	436	370	350	555
Right ventricle	357	197	390	880
Septum	458	354	389	855
Small intestine	312	625	1,433	856
Spleen	400	648	760	1,440
Stomach	344	199	0	457
Testicle	*	172	*	154
Thyroid	301	1,045	222	800
Tongue	472	257	82	119
Trachea	91	56	0	138
Ureter	102	*	133	0
Uterus	114	*	374	*
Abdominal aorta	500	152	0	0
Bile	*	*	0	0
Brachial plexus	0	0	0	0
Brain	0	0	0	0
Carotid artery	0	0	0	0
Ear cartilage	*	*	0	0
Femoral artery	*	*	*	0
Intercostal muscle	38	0	0	0
Jugular vein	0	0	0	0
Marrow	*	*	0	0
Phrenic nerve	0	0	0	0
Pulmonary artery	0	0	0	0
Rib	104	79	0	0
Skull muscle	0	0	50	26
Skin	0	0	0	0
Subclavian artery	*	*	0	0
Subclavian vein	*	*	0	0
Spinal cord	0	0	*	*
Thoracic aorta	0	100	20	0
Tooth	*	*	0	0

*Tissue not examined.

findings are generally in agreement with those reported by other investigators[22-27] if allowance is made for difference in dosage, route of administration, and the time of tissue collection following the injection. In the present study many organs not previously studied for cadmium content were examined. The high concentration of Cd^{115m} in the kidney and gastrointestinal tract is consistent with its routes of excretion. The concentration of Cd^{115m} varied widely among the various tissues.

Although initially the distribution of injected cadmium must depend, in part at least, on local blood supply, the absence of detectable radiocadmium in the brain of each animal indicates blood flow was not the only determinant. This is also substantiated by the absence of Cd^{115m} in the renal medulla two hours after injection and the relatively low concentration in the spleen of each animal. Ultimately the fate of cadmium is determined by factors dependent upon the chemical properties of the element and of the tissues, including successive reactions with compounds having varying degrees of affinity for the metal.

Excretion.—Urine was collected daily during the experiments as spontaneously voided up to the time of sacrifice. Only one animal voided during the first 120 minutes of the experiment, and the sample was free of radiocadmium. A single 24-hour specimen of urine, obtained from one animal prior to sacrifice did not contain Cd^{115m}. Two successive 24-hour urine specimens were collected from each of two animals and were found to be free of the tracer. On two successive days, 24-hour stool specimens were collected from two animals; these were the only samples which could be obtained. They were found to contain considerable quantities of radiocadmium (Table III). Cage washings, obtained daily in all surviving dogs, failed to reveal Cd^{115m}.

TABLE III. FECAL LOSS OF Cd^{115m} IN 2 DOGS IN C.P.M. PER GRAM OF WET STOOL

	SPECIMEN 1 (24 HR.)		SPECIMEN 2 (48 HR.)	
DOG	C.P.M./GM. OF STOOL	TOTAL STOOL CONTENT (C.P.M.)	C.P.M./GM. OF STOOL	TOTAL STOOL CONTENT (C.P.M.)
A	117	24,731	82	13,186
B	244	8,247	603	5,704

Comment.—The accepted routes of excretion of cadmium are through the kidneys and the gastrointestinal tract.[27, 28] Although all stools examined in the present study contained Cd^{115m}, the absence of the detectable radiocadmium in urine samples was consistent. The small amount of radiocadmium administered and its low specific activity were considered to be responsible in part for the lack of detectable quantities of the tracer in the urine. This factor probably was responsible also for some of the tissues being found to be free from the tracer (Table II). The rate of renal excretion of cadmium is normally low, much lower than that for the gastrointestinal tract.

SUMMARY

From 27 to 36 per cent of intravenously injected Cd^{115m} was found to leave the plasmal compartment per minute. During the same period of time,

between one-tenth and one-half of 1 per cent of the tracer in the extraplasmal compartments returned to the plasma.

The rate of disappearance of radiocadmium from plasma was greater than from erythrocytes or whole blood. Initially, the concentration in erythrocytes rose as the plasma concentration fell, then remained approximately constant for 24 to 48 hours.

Cd^{115m} was detected in all stool specimens examined, but none of the urine specimens were found to contain radiocadmium in sufficient concentration to result in counts significantly greater than background.

The distribution of radiocadmium was found to be widespread and quite variable in many tissues of 4 dogs, 2, 24, and 48 hours after injection of the tracer.

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THE EXCRETION AND BIOLOGIC DECAY RATES OF Cd^{115m} WITH A CONSIDERATION OF SPACE, MASS, AND DISTRIBUTION IN DOGS

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PREVIOUS studies in this laboratory[1] have revealed the extremely rapid disappearance of injected radiocadmium from the plasma of dogs, its subsequent widespread distribution in tissues, and slow rate of excretion during the immediate postinjection period. The present report concerns further investigation of the excretion and tissue distribution of Cd^{115m} over much longer periods of time. This is of interest since so little is known of the kinetics of this heavy metal. Furthermore, cadmium has been found in man in trace amounts[2, 3] and has recently been reported to occur naturally in the kidneys of horses[4] and to be related to enzymatic phenomena.[4, 5] Trace quantities of various metals are important in the normal and abnormal states of man.

METHODS AND MATERIALS

Cd^{115m} (half-life, 43 days) was obtained from Oak Ridge National Laboratories as cadmium nitrate in excess nitric acid and neutralized with sodium hydroxide before use. Since the Cd^{115m} was of low specific activity, and in view of the high toxicity of the element, the dosages used were lower than desired for thin mica end window Geiger-Müller tube counting. Four dogs received between 800,000 and 900,000 c.p.m. per kilogram (20 to 50 μc) with 0.32 to 0.40 mg. of cadmium per kilogram of body weight. The fifth dog received 0.36 mg. of the element per kilogram of body weight. Because of physical decay, this contained only 450,000 c.p.m. per kilogram of body weight by the time of its use. No toxic reactions were noted during or following the injections.

The five healthy dogs were placed in separate metabolic cages. For several days prior to injection, the dogs were maintained on the same diet (one pound of Pard* daily), as throughout the experiments. After intravenous injection of the tracer, the dogs were returned to the metabolic cages. Urine and feces were collected each morning, and the bottom pan was rinsed twice daily and assayed for radioactivity. Food and water remaining from the previous 24 hours were removed, measured, and replaced by known amounts. Blood was collected at intervals of 1 to 3 days. The radioactivity of all samples was assayed by methods previously described.[7]

The animals were sacrificed at the end of 20 days (2 dogs), 21 days (2 dogs), and 30 days (1 dog). Specimens were obtained from the organs listed in Table II, washed with water, and blotted dry. They were then placed in weighing bottles, weighed to the nearest tenth of a milligram, and transferred to digestion flasks. Digestion was accomplished with concentrated nitric acid and heating, the excess nitrates being driven off with

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*Canned dog food manufactured by Armour & Company, Chicago, Ill.

formic acid according to the method of Sheppard and Martin.[8] The contents of the flasks were transferred to 5 or 10 ml. volumetric flasks and diluted with distilled water. Counting was done with a thin mica end window Geiger-Müller tube.[7]

Determinations of radiocadmium space were based on the Cd^{115m} content of the erythrocyte and plasma samples collected at 1- to 3-day intervals and expressed as milliliters of plasmal and erythrocyte volume equivalents. Calculations were computed by methods previously described[10] as follows:

1. In accordance with the principle of dilution the amount of tracer in the body was divided by the serum concentration of the tracer expressed by the following equation:

$$\text{Ct}\left(\begin{matrix}\text{equivalents at time t}\\ \text{calculated space in serum}\end{matrix}\right) = \frac{\begin{matrix}\text{amount of tracer administered} -\\ \text{amount excreted by time t}\end{matrix}}{\text{serum concentration at time t}}.$$

2. Based on the same principle space can be determined for the time of injection of t_o, by extrapolating the serum concentration curve after equilibrium of distribution has been reached back to the time of injection (t_o) and dividing the amount injected by the theoretic serum concentration obtained for t_o. This was expressed as follows:

$$S_o = \frac{N_o \text{ (amount of tracer administered)}}{CN_o \text{ (extrapolated serum concentration of tracer at } t_o)}.$$

3. By use of biologic decay rates and the appropriate formulas, Cd^{115m} *space* at any time t was determined in the dogs after essentially a steady state had been reached with the serum concentration of the tracer decreasing at a single exponential rate for at least several days. The t values so obtained were employed as previously described[11] by the following formula:

$$b_t = \frac{\log CN_{t_1}}{CN_{t_2}},$$

where CN_{t_1} = concentration in serum at time t_1, CN_{t_2} = concentration in serum at later time and t_2 and b_t = rate of decrease in serum concentration, i.e., slope of curve of serum concentration of the isotope at time t.

Mass of the nontracer can be determined as previously described[11] from the equation:

$$M = \frac{I}{b_t},$$

where M = exchanging body mass of nontracer at time in grams, and I = daily intake of cadmium in grams.

Furthermore, from the above, the size of the exchanging cadmium compartment can be calculated from the equation:

$$V_t = \frac{M_t}{CM_t},$$

where V_t = Cd^{115m} space at time t, and CM_t = concentration of nontracer in serum at time t in grams per milliliter.

Equilibrium of Distribution.—Cadmium has been reported in trace amounts in animals and in man, previously having been considered a contaminant in the latter. The injected radiocadmium and carrier thus may be considered to represent a relatively large amount of the element for the dogs. In this light, the analogy to radiomercury and mercury in the studies in man[9] is obvious.

Equilibrium of distribution was considered first established following a single intravenous injection of the tracer when the compartments with which the tracer exchanged first developed constant rates of regression in concentration of the tracer. This is surely never fully known since each compartment cannot be sampled. The serum compartment

was used for convenience. This problem has been discussed more fully elsewhere.[12] During the first week of observation the concentration of Cd^{115m} in the plasma fell to zero and the percentage of administered isotope remaining in the body decreased. These findings show equilibrium of distribution never fully to be attained, but essentially a steady state was established. The failure to attain equilibrium of distribution may, to a large extent, be attributed to the fact that the cells of the body constitute the major compartment of radiocadmium. Moreover, the rate of loss from this compartment was considerably slower than the rate of entry into it. Furthermore, these experiments showed the erythrocyte not to be a satisfactory intracellular compartment to reflect the kinetics of intracellular cadmium throughout the body. In spite of retention by the body of approximately 95 per cent of the injected Cd^{115m} during the entire period of study, the erythrocyte content of the tracer slowly diminished to zero over a period of several weeks. This further shows the extremely slow rates of transfer of Cd^{115m} and the magnitude of the extravascular intracellular compartment with subsequent shifts among intracellular compartments. In addition, there was considerable variation in affinity of various cells for cadmium. This affinity may change with age and vary among animals. It most likely is related to tissue proteins which react with cadmium.

Space and Mass.—Because the actual total nontracer in the body was unknown, mass could not be calculated. Space was calculated according to methods previously described, but artifactual variations were obvious and rendered the computations valueless.

The difficulties and inaccuracies associated with determinations of space and mass have been presented previously.[12] However, a few observations are available further to indicate the difficulties and errors involved in estimating space and mass of cadmium as well as other substances from this type of study. Calculations to determine total body space are dependent upon a knowledge of the total quantity of tracer in the body and not on the content of a single sampled compartment unless that compartment is truly a representative mean for the body as a whole and equilibrium of distribution is obtained. Sequestration of the tracer in any compartment renders difficult or impossible, accurate determinations of total space since they are based on the assumption of active and rapid exchange among compartments albeit varying quantitatively from moment to moment.

The sequestration, whether it be anatomic or functional, results in partial or complete, temporary or permanent isolation of a portion of the tracer from the rest of the body. This is also associated with a lag in equilibrium of distribution. If the sequestered compartment is small, its effect is minimal. Should, however, the "blinking" phenomenon[12] involve a large compartment, considerable variations in the calculated space and mass would occur with time. The rate of exchange of such compartments is of considerable importance in the determination of its effect on calculated space and mass.

Extreme variations in calculated space noted in this study, were attributed to a large extent to the lack of proportional change or discordancy between increases or decreases in the rates of radiocadmium excretion and increases or decreases in the tracer content of the sampled compartment. Cadmium does not exist in the body to any extent other than in the tissues. Of course, this holds true for the tracer. Thus, the concepts of space, as generally applied to other elements such as sodium, could not be satisfactorily applied in this investigation. Shifts in body water and its subcompartments were not of great consequence although such phenomena can alter the values of "calculated" space and mass. As noted above, significant errors can arise from asynchronous or disproportionate changes in the tracer content of various compartments of the body. For these reasons, estimation of the space and mass of elements such as cadmium and mercury which are fixed or sequestrated in the tissues must be made by other means than by sampling plasma or erythrocytes.

Cadmium is distributed in some, but not all of the body areas (Table II). The tissue compartments is certainly the largest of these but is not homogeneous itself being of numerous subcompartments of varying size, composition, and activity.

RESULTS

Biologic Decay Rates.—The biologic decay rates observed for the 5 dogs are summarized in Table I. Because of the minimal excretion during the period of observation and the low concentration of the tracer it was not possible to assign definite significance to the calculated values, but rather to submit the data as estimated or approximate values and to show the slow rates of biologic decay of cadmium. It will be noted that only Dog E did not excrete any cadmium in the urine. Dog I passed stools on only 9 of 21 days. The proportional distortion of the estimated biologic decay rates is obvious. These data emphasize the importance of the gastrointestinal route of excretion of cadmium. The importance of knowing the biologic decay rates of cadmium in cadmium poisoning and radiocadmium exposure is obvious.

TABLE I. APPROXIMATE BIOLOGIC HALF-LIVES OF Cd^{115m} (IN DAYS) FOR 5 DOGS

DOG	$U_{½}$	$S_{½}$	$E_{½}$
E	--	419	388
F	2,254	659	500
G	1,273	493	333
H	2,659	426	260
I	3,597	16,922	1,046

$U_{½}$ indicates the time that would be required to excrete half of the administered tracer by way of the urine alone; $S_{½}$ by way of the stools alone; and $E_{½}$ by way of both urine and stools.

If Dog I is ignored, from 419 to 659 days would be required to eliminate one half of the injected tracer by the stools, from 1,273 to 2,659 days to eliminate one half of the injected tracer by way of the urine, and from 260 to 500 days to eliminate one half of the injected radiocadmium by both routes simultaneously. Dog I shows the possible magnitude of individual variations.

Tissue Distribution of Cd^{115m}.—The distribution of radiocadmium in the tissues of 5 dogs many days after administration of the tracer is presented in Table II. The results of previous studies[1] obtained in two dogs sacrificed 2 and 24 hours after the intravenous injection of the Cd^{115m} are also shown. It is apparent that the qualitative distribution is essentially the same in all dogs, discrepancies being noted only in those tissues in which the concentration was generally low. This, when coupled with biologic decay, can well account for the differences noted. Qualitative differences between the same tissues in different dogs were generally of no significance, being manifestations of variations in counting, weighing, preparation, or in rates of biologic decay. However, there was a consistent increase in the percentage content of Cd^{115m} in the renal cortex with the passage of time. The kidney of man has been found by others[3] to contain more cadmium than other organs and a naturally occurring cadmium proteinate has recently been reported in the kidneys of horses.[6] All expressions of tissue cadmium content were corrected to a dosage of 425,000 c.p.m. per kilogram of body weight and the average content was determined for the 7 dogs. As demonstrated in this and previous studies[1] the gastrointestinal tract was the main route of cadmium excretion. Radiocadmium was detected in the bile of only one dog, indicating the excretion to be extrahepatic.

TABLE II. TISSUE DISTRIBUTION OF Cd^{115m} IN C.P.M. PER GRAM OF WET TISSUE IN 7 DOGS FOLLOWING INTRAVENOUS INJECTION (ADJUSTED TO A DOSE OF 425,000 C.P.M. PER KILOGRAM OF BODY WEIGHT)

	DOG STUDIED AND TIME AFTER INJECTION						
TISSUE SITE	C 24 HR.	D 2 HR.	E 30 DAYS	F 20 DAYS	G 20 DAYS	H 21 DAYS	I 21 DAYS
Adrenal	47	202	0	0	226	0	587
Bladder	175	0	13	53	91	0	110
Diaphragm	74	176	0	26	34	79	143
Endomyocardium	239	692	0	23	1,086	138	125
Epimyocardium	478	318	178	77	89	143	168
Esophagus	126	23	169	58	6	34	79
Eyeball	25	53	0	51	15	23	18
Gall bladder	514	380	1,035	426	268	547	366
Intercostal muscle	29	0	0	13	30	25	90
Large intestine	407	539	0	142	70	77	170
Left auricle	377	808	407	0	329	0	387
Left ventricle	405	630	172	62	108	139	201
Liver	10,403	8,892	9,050	2,129	3,909	3,086	7,668
Lung	174	395	314		90	59	42
Marrow				113	142	114	606
Mediastinal node	444	519	48	0	365	48	525
Mid-myocardium	506	520	38	0	146	161	0
Ovary	225				171		
Pancreas	721	4,600	2,095	456	468	563	432
Papillary muscle	180	315	535	35	108	16	98
Pericardium and fat	135	173					
Renal cortex	1,336	2,380	4,939	1,503	2,465	4,655	5,850
Renal medulla	648	0	0	336	413	259	416
Retina	469	80	0	0	200	16	20
Rib	23	0	92	0	84	0	113
Right auricle	350	555	26	43	339	0	289
Right ventricle	390	880	180	189	178	81	159
Septum	389	855	139	0	685	0	46
Skull muscle	50	26	0	0	93	88	0
Small intestine	1,433	856	147	139	122	0	237
Spleen	760	1,440	1,685	345	495	733	845
Stomach	0	457	556	144	16	160	10
Testis		154	92	129		250	114
Thyroid	222	800	155	131	267	54	33
Tongue	82	119	0	39	83	211	90
Trachea	0	138	0	76	78	0	61
Ureter	133	0	0	0	281	0	102
Uterus	374				46		
Ear cartilage	0	0	0	0	102	0	0
Abdominal aorta	0	0	184	162	0	0	0
Bile	0	0	0	58	0	0	0
Brachial plexus	0	0	0	0	0	190	0
Brain	0	0	0	0	0	0	0
Carotid artery	0	0	0	*	*	*	*
Femoral artery	*	0	0	*	*	*	*
Inferior vena cava	0	0	0	0	0	0	0
Jugular vein	0	0	0	0	0	0	0
Peritoneum	*	*	*	0	0	0	0
Phrenic nerve	0	0	0	0	0	0	0
Portal vein	*	*	*	0	0	0	0
Pulmonary artery	0	0	171	0	0	0	0
Subclavian artery	0	0	0	0	0	0	0
Subclavian vein	0	0	0	0	0	0	0
Superior vena cava	0	0	0	0	0	0	0
Skin	0	0	0	0	0	0	0
Thoracic aorta	0	0	0	0	0	0	0

*Tissue not studied.

Most impressive was the wide distribution in magnitude of concentration of radiocadmium 3 and 4 weeks after its administration. This substantiates the duration of retention of cadmium, probably in part at least, in chelated states. This is also of significance in consideration of cadmium poisoning or radiocadmium radiation effects. The movement of cadmium out of the plasma is rapid but the mechanism of transportation, fixation, and excretion remain unknown. Cadmium is not isolated to one single compartment nor in all body compartments, but rather its location varies considerably (Table II). The nature and quantities of these sequestered compounds of cadmium is surely determined by the physiochemical constitution of the respective tissues and their varying chemical affinity for cadmium.

DISCUSSION

The studies show that equilibrium of distribution was never fully attained. The rate of excretion rate of Cd^{115m} was so slow and the specific activity of the tracer material used was so low that body or compartmental kinetics could not be observed as accurately as desired but sufficiently well to show their interesting and peculiar characteristics. Certainly knowledge of the prolonged retention of most of the injected tracer is important as well as is the relative magnitude of the gastrointestinal route of excretion. Moreover, it is obvious that the customary determinations of space and mass could not be applied to Cd^{115m} in these experiments in view of the marked sequestration in many tissue compartments and failure to measure the infinitesimal amounts of the nontracer.

The mechanism by which cadmium is excreted primarily by the gastrointestinal tract and relatively poorly by the kidneys must be related to many of the unknown and interesting aspects of heavy metal metabolism such as iron, mercury, cobalt, and others. Although the concentration of the Cd^{115m} was high in the tissues of the kidney and liver, little was excreted in the urine and bile indicating firm fixation of the tracer in the cells of at least these two organs. These phenomena need investigation and Cd^{115m} lends itself readily for such studies. It has many fine characteristics for tracer research.

The slow rate of excretion of cadmium emphasizes the importance of the physical half-life of the radioactive isotopes in estimating radiation hazard. The rate of the excretion is less significant. However, methods may be developed to increase considerably the rate of excretion and thereby shorten the biologic half-life of the tracer.

SUMMARY

The biologic half-life of Cd^{115m} is long, due to the relatively slow rate of excretion of the element. The most rapid route of excretion was in the feces. Because of its great tendency to sequestration and unequal distribution among tissues of the body, space and mass of cadmium could not be satisfactorily estimated.

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CLINICAL AND LABORATORY FEATURES OF TWO VARIANTS OF METHEMOGLOBIN M DISEASE

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METHEMOGLOBINEMIA, as a cause of cyanosis, was first recognized by Slosse and Wybauw.[1] There were few further studies of this disease until Hitzenberger[2] and Dieckmann[3] each described a patient. Since then, about 100 instances of hereditary methemoglobinemia have been described in the literature. In 4 families studied, it appeared to be a mendelian dominant characteristic, whereas in one it was recessive.[4]

In hereditary methemoglobinemia, hemoglobin iron is present in the ferric state which implies a structural defect on the heme portion of the hemoglobin molecule. Furthermore, there is failure of methemoglobin to revert to hemoglobin when blood from a patient with hereditary methemoglobinemia is allowed to incubate in the presence of glucose in vitro.[5] This biochemical lesion has been attributed to an inherited lack of "methemoglobin reductase," and a resulting inability to reduce constantly forming methemoglobin to functioning hemoglobin[6-8] which then allows an intracorpuscular accumulation of methemoglobin. The stability of this compound leads to faulty oxygen transport and thence to cyanosis. However, it has become recently apparent that even this rare disease may result from different pathogenic mechanisms.

A globin abnormality leading to faulty oxygenation of hemoglobin was found by Hörlein and Weber[9] in a German family with a history of hereditary cyanosis for 4 generations. The protein abnormality was suspected by an abnormal spectrophotometric absorption spectrum of the patient's hemoglobin, which exhibited an unusual maximum near 600 mμ. When the heme and globin moieties from patients and from normal subjects were split and recombined with each other, the abnormal spectrophotometric pattern persisted in the new hemoglobin, which contained the patient's globin and normal heme, proving that the defect was of protein origin. This type of hemoglobin, designated by Singer[10] as type M was further characterized by starch electrophoresis and by spectrophotometry as an abnormal methemoglobin.[11, 12] Erythrocytes which contained this pigment were found incapable of reducing nitrite-induced methemoglobin to hemoglobin.[13] Furthermore, the abnormal methemoglobin crystallized in ferric form.[14]

Since 1953, we have studied two families with a similar type of hemoglobin defect. Our studies have brought out further differences between the

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characteristics of methemoglobin M encountered in the two families. It now appears that at least two and possibly more types of methemoglobin M exist. Of incidental interest is the presence of a second type of abnormal hemoglobin in Family II, as yet unidentified, with electrophoretic characteristics of type C. The purpose of this paper is to provide additional information relative to the clinical characteristics of this disease, the spectrophotometric properties of the hemoglobin, and the ability of the intact erythrocytes from these patients to reduce methemoglobin in vitro.

METHODS

A precise quantitative determination for methemoglobin M is not available as the molecular extinction coefficient for this substance is not as yet known. For this reason, an approximation of the quantity of methemoglobin M was carried out by several methods. In the first of these, methemoglobin was determined from the quotient of the values for optical density of a 1:100 hemolyzate of whole blood in M/60 phosphate buffer, pH 6.6 at 576 mμ and at 510 mμ.[15, 16] The per cent of methemoglobin was determined from a straight line which connected O.D. $\frac{576}{510}$ in a normal hemoglobin, free of methemoglobin by spectrophotometric analysis (methemoglobin 0 per cent) and the same hemoglobin, treated with an excess (0.1 ml.) of 10 per cent potassium ferricyanide (methemoglobin 100 per cent). It was assumed for this approximation, that the relationship of O.D. $\frac{576}{510}$ at 0 and 100 per cent methemoglobin was linear. In addition, methemoglobin was determined by the procedure of Evelyn and Malloy,[17] as the difference in optical density at 630 mμ of a 1:100 dilution of whole blood in M/60 phosphate buffer, pH 6.6, before (D1) and after (D2) the addition of neutralized potassium cyanide. The amount of methemoglobin determined as grams per 100 ml. was expressed as per cent of total cyanmethemoglobin determined by the absorption density at 540 mμ of a 1:500 dilution of cyanmethemoglobin (D3). Methemoglobin was also determined from the above data, by the ratio of the values D3/D1 and was expressed as per cent of total hemoglobin. This was also standardized against a straight line which connected K $\frac{D3}{D1}$ for 0 and 100 per cent methemoglobin, respectively. All determinations were performed in a Beckman DU spectrophotometer standardized against the mercury line at 446 mμ. Hemoglobin was standardized by the Wong[18] iron method. Absorption spectra under various experimental conditions were determined in the Beckman DU spectrophotometer on whole blood diluted 1:100 with buffer. Filter paper electrophoresis was accomplished on the Grassman-Elphor horizontal chamber, utilizing 0.05 M barbital buffer, pH 8.6 at 300 volts for 8 hours. Starch electrophoresis was performed according to the method of Kunkel and Wallenius.[19]

Methemoglobin was produced in intact washed erythrocytes by incubating one part of packed erythrocytes with 1.5 parts of sodium nitrite (5 mM. per 1,000 ml. of 0.85 per cent NaCl) for 2 hours. The rate of formation of methemoglobin was determined by observations every 10 minutes for the first hour and every 15 minutes the second hour. The rate of reduction of methemoglobin was determined by suspending the nitrite-treated washed erythrocytes in glucose or plasma. Methemoglobin was determined every hour for 5 hours and in 24 hours.

CASE REPORTS

Family I.—Case 1A. The propositus, Antoinette V., a 25-year-old white woman of Italian origin, was cyanotic as an infant. However, the diagnosis of congenital heart disease could not be confirmed by repeated physical examinations and roentgenographic observations. She showed normal childhood development and, with the exception of the

usual childhood diseases, suffered no incapacitation. The diagnosis of congenital methemoglobinemia was first made at the time of her marriage. Treatment with ascorbic acid and with methylene blue was attempted, but was not successful. The patient was asymptomatic with the exception of moderate fatigue on exertion, but denied dyspnea, vertigo, syncope, or headaches. There was no history of drug ingestion, and there were no gastrointestinal symptoms. The most striking physical finding was a bluish-slate gray tinge to the lips, skin, and nail beds. The lungs were clear, the heart slow and regular, no murmurs were heard, and the blood pressure was 110/70. The liver and spleen were not palpable.

Hematologic examination showed the following: hemoglobin 13.5 Gm. per 100 ml.; R.B.C. 4.93 million per cubic millimeter; C.I. 0.89; W.B.C. 8,800 per cubic millimeter with 56 per cent segmented neutrophils; band forms 7 per cent; lymphocytes 28 per cent; monocytes 5 per cent; eosinophils 1 per cent; and basophils 3 per cent. Reticulocytes numbered 3 per cent and the wet smear showed no Heinz-Ehrlich inclusion bodies.

CASE 1B. Donald V., 4 years old when first seen, son of the propositus, was cyanotic at birth. Physical examination, electrocardiogram, and roentgenographic examination failed to disclose congenital heart disease. With the exception of pneumonia in May, 1952, this boy had been completely normal and active. On physical examination, there was a bluish tinge to the skin, lips, and nail beds. The lungs were clear. The heart disclosed no murmurs, enlargement, or irregularity. The liver and spleen were not palpable. Treatment with ascorbic acid and methylene blue had no effect upon the cyanosis.

Hematologic examination showed a hemoglobin value of 14 Gm. per 100 ml. The red cells numbered 5.40 million per cubic millimeter and the leukocytes 9,700 per cubic millimeter. The differential formula was entirely normal with the exception of an increase in the number of basophils to 4 per cent. Reticulocytes numbered 2 per cent. There were no inclusion bodies seen in the wet smear.

CASE 1C. Michael V., son of the propositus, was apparently normal at birth. He developed normally during the first few weeks of infancy. At the age of 6 weeks, cyanosis was first noted. At first, this occurred following sustained periods of crying, but later, cyanosis persisted. Physical examination disclosed no abnormalities with the exception of cyanosis of the nail beds and mucous membranes. Hematologic examination was not significant.

The remainder of the family history is depicted in Fig. 1. A third son has remained normal. The woman's parents, siblings, and husband showed no abnormality.

Family II.—CASE 2A. The propositus, A. P., a white woman of Swiss parentage, was 33 years old when first seen. She was born cyanotic and was mistakenly thought to have congenital heart disease, but was always fully active. She had never received ascorbic acid or methylene blue. Physical examination showed a grayish cyanotic hue of the lips, nail beds, and skin. There was a soft Grade 2 systolic murmur, located at the third intercostal space, left of the sternum. The spleen was palpable 4 cm. below the costal margin. Hematologic examination showed the following: R.B.C. 4.26 million per cubic millimeter; hemoglobin 12.5 Gm. per 100 ml.; leukocytes 11,450 per cubic millimeter; platelets 166,000 per cubic millimeter; and reticulocytes 5.3 per cent. Differential count was not remarkable. No target cells were present. The wet smear was negative for sickle cells and for Heinz-Ehrlich bodies.

Alkali denaturation showed 14.6 per cent fetal hemoglobin in venous blood, and 18.9 per cent in arterial blood. A paper electrophoretic pattern of the stroma-free hemoglobin filtrate (Fig. 2) showed two hemoglobin components; a rapid dark-brown spot, which had the mobility of type A hemoglobin, and a slowly migrating bright-red hemoglobin spot which moved in the vicinity of type C hemoglobin and was tentatively identified as type "C." Mechanical fragility of intact erythrocytes was 4.0 per cent. Osmotic fragility studies showed beginning hemolysis at 0.48 per cent saline and complete hemolysis at 0.32 per cent saline. Total serum bilirubin was 1.8 mg. per 100 ml., all in the indirect reacting form. A 4-day stool specimen showed an average 24-hour excretion of 773 mg. of urobilinogen. Half-life of the patient's Cr^{51}-labeled erythrocytes was 20 days (normal $T\frac{1}{2} = 25$–35 days). There was no predominating sequestration of erythrocytes by liver or spleen.

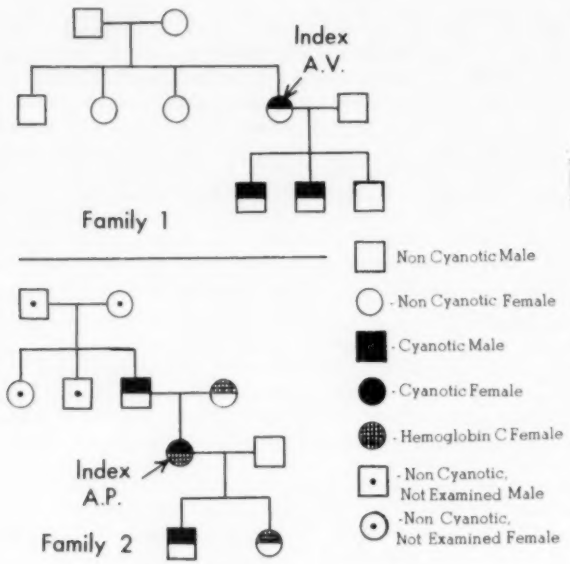


Fig. 1.—Family trees. Families I and II methemoglobin M disease. The half-filled squares or circles designate the heterozygous state.

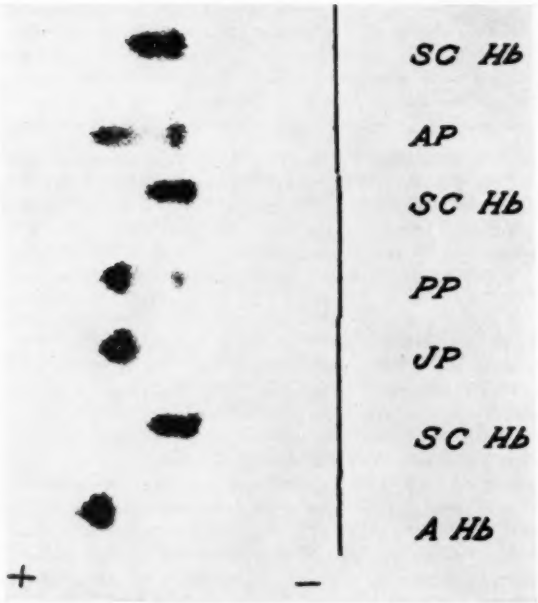


Fig. 2.—Paper electrophoretic patterns of hemoglobins. S.C. = Sickle cell hemoglobin disease. A. P. = Patient 2A (propositus). P. P. = Patient 2C (daughter). J. P. = Patient 2B (son). A = Normal (adult) hemoglobin.

CASE 2B. J. P., the son of A. P., 20 months old when first seen, was apparently normal at birth. Cyanosis was first noted at 11 months of age, during an upper respiratory infection and persisted. He has been completely active and has developed normally. Physical examination disclosed cyanosis of the hands, lips, nails. There was a Grade 1 systolic murmur at the cardiac apex. The spleen was not palpable. Laboratory studies were as follows: hemoglobin 13 Gm. per 100 ml.; erythrocytes 3.90 million per cubic millimeter; leukocytes 19,600 per cubic millimeter; reticulocytes 4.0 per cent; and platelets 282,000 per cubic millimeter. The differential count was normal. Occasional target cells were seen. Mechanical fragility was 6.1 per cent. Alkali denaturation showed 5.7 per cent fetal hemoglobin. Paper electrophoresis disclosed one deep-brown spot which moved with the mobility of adult hemoglobin.

Family Studies (Fig. 1).—A second daughter (P. P.) has remained free of cyanosis and shows no splenomegaly. The hemoglobin was 12 Gm. per 100 ml.; red cell count 5.58 million per cubic millimeter; and reticulocytes 0.2 per cent. Many target cells were present. Paper electrophoresis disclosed two bright-red components: one which migrated as type A and the other as type C. Fetal hemoglobin was 0.5 per cent. Methemoglobin was not present.

The patient's father (J. Z.) is known to have cyanosis since the age of 2. At one time, he was given methylene blue with no relief of cyanosis. Electrophoretic pattern of the father's blood showed one type of hemoglobin, which had a brown color and migrated in the same vicinity as type A. The fetal hemoglobin value was 2.4 per cent. He had one brother and one sister, believed to be normal. Both of his parents were not cyanotic.

The patient's mother (A. Z.) was not cyanotic. Electrophoresis of her hemoglobin on paper showed two distinct bright-red spots. One of these had the mobility of type A hemoglobin, the other had the mobility resembling type C hemoglobin. Thus, it was apparent that the index case of Family II, A. P., was a double heterozygote who showed 3 types of hemoglobin—type M and F transmitted from the father and type C from her mother.

RESULTS

The amount of methemoglobin in the various members of the families as tested by these methods will be found in Table I. It was possible to demonstrate methemoglobin by the Evelyn-Malloy technique in the affected members of Family I (A. V., D. V., and M. V.) since potassium cyanide eliminated the methemoglobin peak. Comparison of methemoglobin values determined by $K\frac{576}{510}$, the Evelyn-Malloy procedure, and by $K\frac{D3}{D1}$ showed agreement within 3 per cent.

On the other hand, in the affected members of Family II (A. P., J. P., and J. Z.) there was no difference in the optical density of the red cell hemolyzate before and after the addition of potassium cyanide, and the amount of methemoglobin estimated by this method was apparently zero. However, the determination of methemoglobin by $K\frac{576}{510}$ confirmed that about 27 and 20 per cent of the pigment in A. P. and J. R., respectively, was methemoglobin. The inability of potassium cyanide to eliminate completely the absorption density at 630 mμ, probably explains why this pathologic hemoglobin cannot be accurately determined by the procedure of Evelyn and Malloy in Family II.

Absorption Spectrum Studies.—The absorption spectrum of a hemolyzate of whole blood in buffer was determined at various pH levels (Figs. 3 and 4). At pH 8.3 (Figs. 3, *B* and 4, *B*) prominent peaks, identical to those found in

TABLE I. METHEMOGLOBIN VALUES BY VARIOUS METHODS IN PATIENTS AND RELATIVES

CASE NUMBER	PATIENT	RELATION	TREATMENT OF R.B.C.	D1*	D2	D3	MHB. (GM. %)	TOTAL HB. (GM. %)	MHB. (%)	MHB. % $K \frac{D3}{D1}$	MHB. % $K \frac{576}{510}$	FETAL HB. %
1A	A. V.	Index	Fresh	0.150	0.077	0.161	3.9	11.8	33	31	30	0
1B	D. V.	Son	Fresh	0.139	0.073	0.172	3.5	12.6	28	26	26	0
1C	M. V.	Son	Fresh	0.159	0.079	0.171	3.6	12.6	30	31	29	0
1D	J. V.	Son	Fresh	0.041	0.047	0.197	0	14.5	0	0	0	0
1E	J. M.	Mother	Fresh	0.037	0.038	0.193	0	15.5	0	0	0	0
1F	J. M.	Father	Fresh	0.038	0.039	0.213	0	15.6	0	0	0	0
1G	L. A.	Sister	Fresh	0.033	0.034	0.174	0	12.8	0	0	0	0
1H	S. M.	Brother	Fresh	0.032	0.034	0.169	0	12.4	0	0	0	0
1A	A. V.	Index	Nitrited	0.228	0.069	0.108	8.5	8.5	100	100	100	-
2A	A. P.	Index	Fresh	0.149	0.143	0.158	0.3	11.6	2.8	25	31	14.6
2B	J. P.	Son	Fresh	0.113	0.107	0.159	0.3	11.7	2.7	23	22	5.7
3C	P. P.	Daughter	Fresh	0.043	0.039	0.166	0.2	12.2	1.7	4	4	0.5
3D	A. Z.	Mother	Fresh	0.049	0.081	—	0	—	-	-	-	0
3E	J. Z.	Father	Fresh	0.117	0.126	—	0	—	-	-	-	2.4
2A	A. P.	Index	Nitrited	0.148	0.096	—	2.8	—	-	-	85	-
Normal		—	Fresh	0.043	0.043	0.210	0	15.4	0	0	0.4	0
Normal		—	Nitrited	0.195	0.056	0.115	7.4	8.4	89	86	88	0

*D1 = Optical density of whole blood in M/60 phosphate buffer, pH 6.6, 1:100. D2 = Optical density of above following addition of 0.1 ml. 10 per cent neutral potassium cyanide. D3 = Total cyanmethemoglobin (1:500).

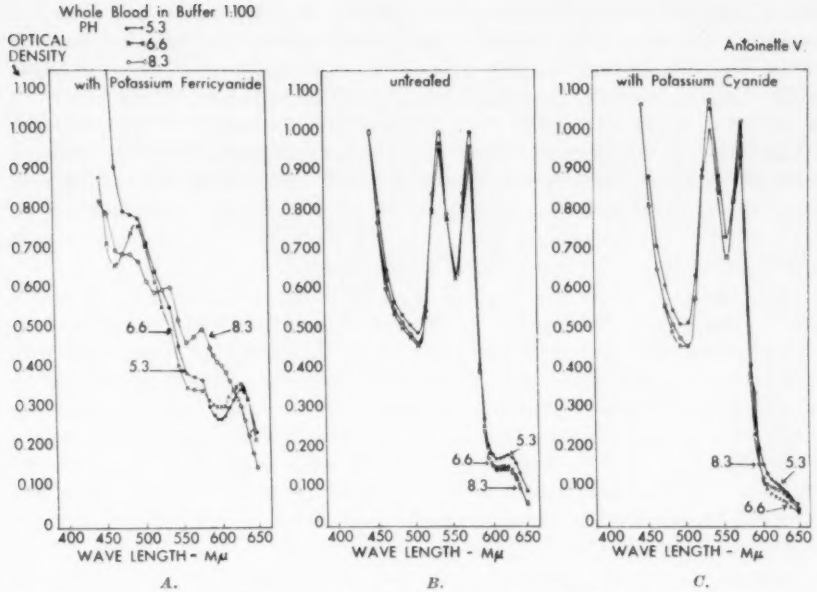


Fig. 3.—Absorption spectra. Hemoglobin of Patient A. V. (Family I) under various experimental conditions.

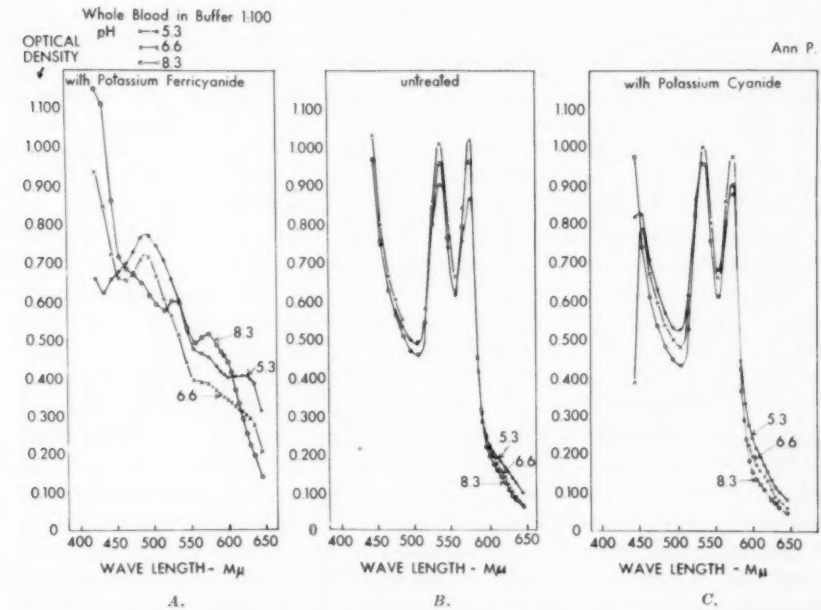


Fig. 4.—Absorption spectra. Hemoglobin of Patient A. P. (Family II) under various experimental conditions.

normal hemoglobin were found at 540 and 577 mμ. Even at this pH level, a prominent difference in spectrophotometric pattern was demonstrable between normal subjects, A. V. (Family I) and A. P. (Family II). Normal hemoglobin (not illustrated) shows no bulge, peak, or inflection in the range between 600 and 650 mμ. A. V. (Fig. 3, *B*) showed a prominent bulge between 600 and 625 mμ with a peak at 620 mμ, which became even more pronounced as the pH was lowered. On the other hand, A. P. (Family II) showed scarcely more than a slight inflection at 615 mμ at pH 8.3, which became a more decided bulge at pH 5.3 (Fig. 4, *B*). This took the appearance of a decrease in slope between 600 and 620 mμ as the pH was lowered.

The effect of potassium cyanide upon the absorption spectrum of pathologic methemoglobin is depicted in Figs. 3, *C* and 4, *C*. Methemoglobin derived from normal blood by treating normal erythrocytes (not illustrated) with nitrite shows an absorption peak at 630 mμ which was virtually eliminated by the addition of potassium cyanide solution. On the other hand, it seems apparent from a comparison of Figs. 4, *B* and 4, *C*, that only partial elimination of the absorption peak had occurred in A. P., while in A. V. (Figs. 3, *B* and 3, *C*) the methemoglobin peak was eliminated by potassium cyanide. This was confirmed by the Evelyn-Malloy procedure, by which it was possible to measure methemoglobin in Family I, but not in Family II.

Methemoglobin was produced from normal hemoglobin by the addition of an excess of an oxidizing agent, 10 per cent potassium ferricyanide which eliminated the deep absorption bands at 540 and 577 mμ. In normal blood (Fig. 5), a sharp peak was produced at 630 mμ that was further emphasized by a rather deep minimum at 600 mμ. A second methemoglobin peak was readily detected at 500 mμ. A. V. (Case 1A), showed a rather sharp peak at 625 mμ, sharply set off by a deep minimum at 600 mμ. In contrast, the absorption peak observed in the blood of A. P. (Case 2A) occurred at 605 mμ. There was only a slight inflection at 600 mμ, which gave the appearance of a plateau between 600 and 625 mμ in this spectral pattern. These characteristics are similar to those reported by Gerald as methemoglobin M_B.[20] In contrast, the differences in the spectrophotometric hemoglobin pattern of A. V. establish this as a unique spectrophotometric pattern.

The presence of two types of hemoglobin of different electrophoretic mobilities in Patient A. P. afforded an opportunity to compare spectrophotometric properties of both hemoglobins after starch electrophoresis eluted into phosphate buffer, pH 6.6. The resulting fractions, a rapidly migrating brown one (fraction III) and a slower bright-red fraction (fraction I) were subjected to spectrophotometric analysis. An intermediate hemoglobin band (fraction II) could not be isolated in sufficiently pure solution for accurate analysis. The plateaulike peak at 620 mμ characteristic of methemoglobin M was present in the hemoglobin derived from fraction III, but not in the bright-red hemoglobin, fraction I. Determination of the relative quantity of methemoglobin M (fraction III) by starch electrophoresis and elution, showed this pigment to be present as 37 per cent of total hemoglobin.

In Vitro Methemoglobin Reducing Studies.—There was essentially little decline in the concentration of methemoglobin in nitrite-treated normal corpuscles incubated in saline over a 24-hour period. However, when the nitrite-treated erythrocytes were suspended in plasma or glucose, methemoglobin was completely reduced to hemoglobin in 24 hours. Erythrocytes from patients with methemoglobin M suspended in normal saline, glucose, or plasma showed no change in the concentration of methemoglobin as compared to

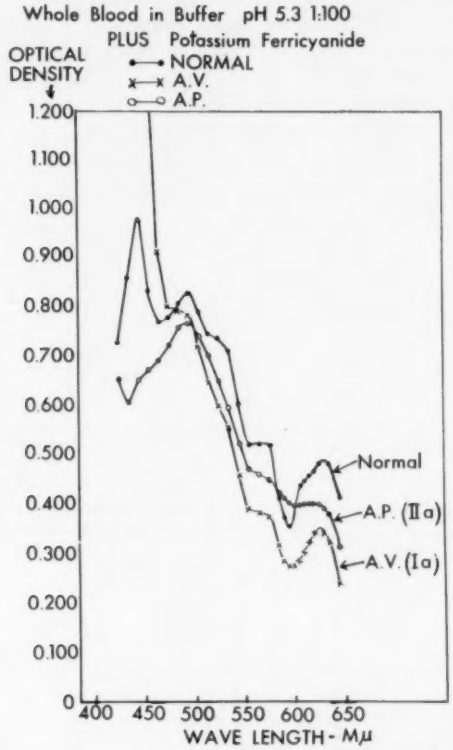


Fig. 5.—Comparison of absorption spectra of normal methemoglobin and both pathologic methemoglobins at pH 5.3.

normal. Treating methemoglobin M erythrocytes with sodium nitrite in the manner described previously resulted in a great intracorpuscular content of methemoglobin. A gradual diminution in concentration of methemoglobin occurred when the patient's nitrite-treated erythrocytes were resuspended in plasma, so that in 24 hours an equilibrium was established with the methemoglobin concentration at the original prenitrite level (Fig. 6). This finding demonstrates conclusively a methemoglobin reducing mechanism in the erythrocytes of the affected members of the families.

The same experiment was performed with Patient M. V. (Case 1C) shortly after birth and again at 6 months of age (Fig. 7, *A*). Blood obtained from

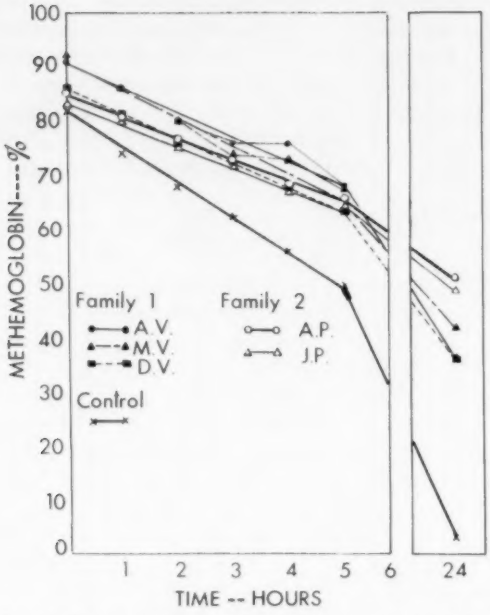
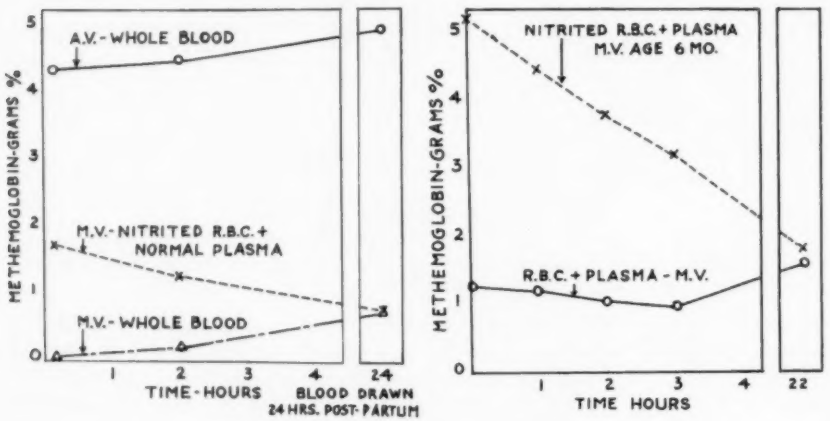


Fig. 6.—Rate of reduction of methemoglobin in erythrocytes treated with sodium nitrite, in normal subjects and patients with methemoglobin M disease.



A. *B.*

Fig. 7.—*A*, Rate of reduction of nitrite-treated erythrocytes from umbilical cord blood of Patient M. V. *B*, Rate of reduction of nitrite-treated erythrocytes (M. V.) at 6 months of age.

the umbilical cord at birth showed a methemoglobin concentration of 0 Gm. and total hemoglobin was 14.8 Gm. These nitrite-treated erythrocytes showed a concentration of 1.66 Gm. per 100 ml. which fell to 0.86 Gm. per 100 ml. after 24 hours' incubation in plasma. On the other hand, the baby's untreated methemoglobin-free erythrocytes, showed a spontaneous increase in methemoglobin from 0 to 1.07 Gm. per 100 ml. during the same period of time. The concentration of fetal hemoglobin was 75 per cent at the time of these studies. Six weeks later, intermittent cyanosis was noted during periods of crying. The methemoglobin concentration was now 1.02 Gm. or 18.1 per cent of total hemoglobin. At the age of 6 months, the infant's nitrite-treated erythrocytes showed a methemoglobin content of 5.2 mg. per 100 ml. immediately after reconstitution in his plasma. In 22 hours of incubation at 37° C., the concentration of methemoglobin fell to 1.8 mg. per 100 ml. in nitrite-treated red cells and 1.6 mg. per 100 ml., in untreated erythrocytes (Fig. 7, *B*). At the time of these studies, there was an absence of alkali-resistant hemoglobin.

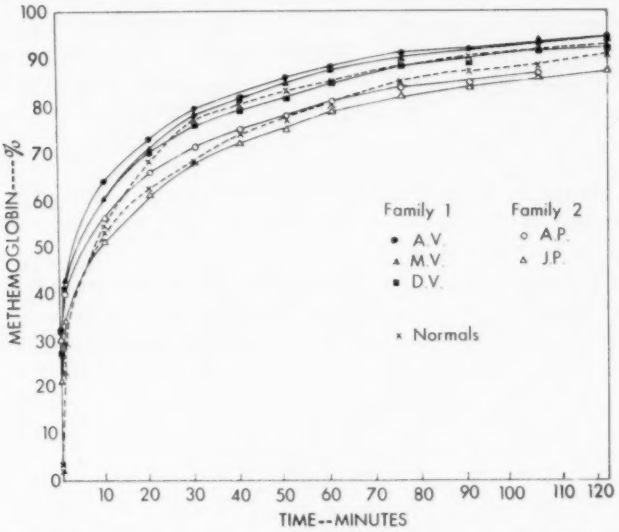


Fig. 8.—Rate of production of methemoglobin by incubation of intact erythrocytes and sodium nitrite.

A methemoglobin reducing mechanism in the erythrocytes of these patients was now known to be present. The rate of formation of methemoglobin was then determined in vitro. Methemoglobin determinations were done every 10 minutes and were expressed as per cent of total hemoglobin. The results are depicted in Fig. 8. The rate of formation of methemoglobin in the patient's erythrocytes was the same as that observed in control red corpuscles. However, 100 per cent of the hemoglobin was converted to methemoglobin more quickly because more methemoglobin was present at the start of the experiment.

DISCUSSION

The patients discussed in this communication represent heterozygous examples of the newly recognized abnormality, methemoglobin M. There is sufficient evidence to state that in methemoglobin M disease, the fundamental abnormality exists in the structure of hemoglobin itself, as opposed to the biochemical defect known to be present in hereditary methemoglobinemia.

Direct studies on the erythrocytes from Patient A. V. (Case 1A) showed that methemoglobin reductase* was present in normal quantity as were glucose-6-phosphate dehydrogenase, aldolose, and glutathione reductase.† Furthermore erythrocytes from our patients showed a limited but definite methemoglobin reducing property, capable of reducing artificially induced methemoglobin to a 35 or 40 per cent prenitrization level. Beyond this, it is impossible to reduce methemoglobin further, either spontaneously or by the use of direct reducing agents both in vivo and in vitro. The constant day-to-day 25 to 35 per cent level of methemoglobin in all the patients studied, even following treatment with methylene blue, suggests that part of the hemoglobin is incapable of being changed further to normal hemoglobin. It is suggested that this stability is due to a genetically determined heterozygous hemoglobin defect. This may find circumstantial support in the observation that the hemoglobin defect was not present in early infancy, possibly because of the presence of fetal hemoglobin, but increased as the fetal hemoglobin values diminished and finally disappeared. This was demonstrated in Patient M. V. (Case 1C) and inferred by history in the members of Family II (J. Z., A. P., and J. P.).

Our observations suggest that methemoglobin M disease is a dominant characteristic which may have started as a mutation in Patient A. V. (Family I). However, it cannot be ruled out that a possible recessive methemoglobin inhibitor gene might have inhibited the defect in the parents and siblings of Patient A. V. (Case 1A) so that the disorder was not apparent in these individuals. This was not a problem in Family II where the disorder was traced as a dominant, heterozygous characteristic to the father of index Case 2A (Patient A. P.). Of additional incidental interest was the appearance of a second hemoglobin abnormality in Patient A. P., derived from her mother. This abnormal hemoglobin, although unidentified, showed the electrophoretic characteristics of C hemoglobin and was responsible for a mild hemolytic anemia and splenomegaly present in Patients A. P., P. P., and presumably in J. Z., but not in the others. The demonstration of a second hemoglobin defect proves conclusively that methemoglobin M was heterozygous in this family and that the methemoglobin M gene is an allele to type A hemoglobin. It may be further stated that methemoglobin M is not a cause of hemolytic disease per se, but is responsible solely for cyanosis.

*We are indebted to Drs. Clement A. Finch and Frank Huennekens, who performed an assay for methemoglobin reductase in this patient.

†We are indebted to Dr. Robert Kellermeyer who provided these enzyme assays in Patient A. V.

Our previously published abstracts on the congenital cyanotic diseases secondary to abnormal hemoglobin are at present subject to revision due to new information gathered over the past year and reported herein (Table II).

TABLE II. SUMMARY OF CHARACTERISTICS DISTINGUISHING BETWEEN NORMAL METHEMOGLOBIN M_B AND METHEMOGLOBIN M_M IN M/15 PHOSPHATE BUFFER, pH 5.3

	MAXIMA mμ	MINIMA mμ	EFFECT OF KCN	FETAL HB.
Normal methemoglobin	630, 500	595	Reduces	0
M_B	605, 495	None	None	Elevated
M_M	625, 495	595	Reduces	0

Family I, previously reported as hereditary methemoglobinemia,[21] has been determined to have a unique type of methemoglobin M. There is partial ability to reduce methemoglobin to hemoglobin in vitro. The absorption spectrum is different from Gerald's methemoglobin M[11, 20] in that absorption maxima appear at 500 and 625 mμ. Potassium cyanide almost completely removed the absorption peak at 625 mμ. There was no fetal hemoglobin demonstrable. Family II represents an entirely different type of methemoglobin M[22] and shares similar characteristics with that described by Gerald.[11, 20] Treatment of hemolyzates from members of this family with potassium cyanide showed no effect upon the absorption peak at 630 mμ and prompted us to state that methemoglobin could not be demonstrated by chemical means. The plateaulike absorption maxima at 605 and an absence of the 600 mμ minimum correspond with that described by Gerald. It has been proposed[23] that methemoglobin M from Family I be called M_M (methemoglobin Milwaukee type) while that of Family II exemplified type M_B (methemoglobin Boston type). A third variety (M_S), has been referred to[20] in a family from Saskatoon, Saskatchewan, Canada. It seems likely that further differences in methemoglobins will be brought out as more families are studied.

The presence of a second electrophoretically slower hemoglobin component in Patient A. P. brought out an extremely unusual double heterozygote composed of methemoglobin M derived from her father and an unidentified bright-red hemoglobin, possibly C, transmitted by her mother and which reappeared as a heterozygous characteristic in her daughter (P. P.). An additional point of difference in both types of methemoglobin lies in the presence of fetal hemoglobin in Family II, but not in Family I. Elevated values for alkali resistant hemoglobin were found in the hemolyzates of Patients J. Z., A. P., and J. P. who also had type M hemoglobin. On the other hand, Patient A. Z. (the mother of A. P.) had type A and type C hemoglobin, but showed no fetal hemoglobin.

It seems well established that methemoglobin M will result in clinical cyanosis but not in the hemolytic syndrome. On the other hand, the presence of splenomegaly and moderate hemolysis in Patient A. P. was attributed to the second slower moving type C hemoglobin.

SUMMARY AND CONCLUSIONS

1. Two families with a hereditary form of cyanosis were shown to have an abnormal form of methemoglobin, present in about 30 per cent of total hemoglobin. There was a dominant hereditary pattern.

2. Both methemoglobins showed an abnormal spectrophotometric curve, differences in their reaction with potassium cyanide, and differences in fetal hemoglobin content.

3. Erythrocytes of affected members of both families contained methemoglobin reductase by direct and indirect assay. There was no difference in the rate of oxidation of hemoglobin to methemoglobin in the intact erythrocytes of normal subjects and of the patients.

4. The delayed appearance of cyanosis until 6 weeks to 6 months after birth, suggests that fetal hemoglobin delays appearance of cyanosis until its disappearance allows the accumulation of methemoglobin M.

5. Methemoglobin M does not cause hemolytic disease. On the other hand, a second unidentified hemoglobin, resembling type C, was responsible for hemolysis in several members of one of the families studied.

6. It has been proposed that the methemoglobin variants be tentatively named M_B (Boston type) and M_M (Milwaukee type) after the cities where they were studied.

We wish to thank Dr. Park S. Gerald for many suggestions made during the course of this study. Dr. Clement A. Finch and Dr. Frank Huennekens performed the direct assay for methemoglobin reductase in Case 1A, and Dr. Robert W. Kellermeyer performed the erythrocyte enzyme studies in the same patient.

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THE EFFECTS OF THEOPHYLLINE ETHYLENEDIAMINE (AMINOPHYLLINE) ON THE CORONARY HEMODYNAMICS OF NORMAL AND DISEASED HEARTS

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IN VIEW of the widespread use of xanthine derivatives and the lack of agreement concerning their value in coronary arterial disease, a study was undertaken to evaluate further the effect of such a drug on the heart.

In the experimental animal it has been said that xanthines act as coronary vasodilators.[1-3] Much of the evidence derives from "open-chest" and "isolated heart" preparations with their attendant advantages and drawbacks. An exception is the study of Foltz and his colleagues[4] which was carried out in anesthetized intact dogs. He found no significant change in the left-sided coronary flow.

Early work concerning the effect of theophylline in normal man was done by Starr and associates[5] in 1938; further observations (using cardiac catheterization) were reported by Howarth and her colleagues.[6] A later study extending the field of study to the pulmonary vascular bed was carried out by Zimmerman,[7] and by Storstein, Helle, and Rokseth.[8]

While claims have been made that xanthines act as coronary vasodilators in the human, this evidence derives indirectly from clinical studies such as those of Brown and Riseman[9] and Bakst and co-workers[10] who felt that aminophylline offered some help to patients with angina. The opposite view was held by Evans and Hoyle[11] and Master and associates.[12]

Thus, by the middle of the century, opinion seemed fairly divided upon the efficacy (in clinical situations) of aminophylline as a coronary vasodilator. Much difficulty centered upon the investigative method used, and the interpretation of the patient's subjective complaint of angina. Later studies with good statistical treatments[13, 14] seemed more accurate and suggested that xanthine derivatives did not reduce the number of angina attacks and, by inference, did not act as coronary vasodilators. In addition, Merrill[15] noted cardiac pain and sudden death upon giving a xanthine to a patient with coronary thrombosis. Other similar reports exist[16]; however, it is fair to say that xanthines have been used frequently in similar circumstances without serious side effects.

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It can be seen that no final answer is so far evident concerning the real effects of a xanthine on the human coronary circulation. Therefore, the results of this investigation are presented.

MATERIALS AND METHODS

Subjects.—The subjects are divided into 3 groups, identified below, and so designated in the tables.

Group 1: Fourteen persons, clinically and radiologically normal, drawn from the laboratory personnel and medical student population.

Group 2: Seven patients with "pure" mitral stenosis, clinically and radiologically typical and confirmed at later surgery.

Group 3: Six patients with cor pulmonale of varying degree and two with myxedema associated with cardiomegaly.

The ages of Groups 1 and 2 were not statistically different. Group 3 fell into an older age group.

Methods.—The Fick principle was utilized in the determination of cardiac output and the nitrous oxide saturation method[17, 18] was used to determine coronary blood flow. The reproducibility of this method in this laboratory has been previously described[19] and recently confirmed.[20]

On completion of the control measurements, 250 mg. of theophylline ethylenediamine (aminophylline) was diluted in 20 ml. of saline and injected into the coronary sinus catheter over a 20-minute period. Coronary blood flow was then remeasured and the catheter quickly (within 2 minutes) placed in the pulmonary artery and the cardiac output measured again. In all studies, the "experimental" coronary blood flow and cardiac output estimations were completed by 17 minutes following the complete injection of aminophylline. The usual formulas were used for calculation of cardiac works and peripheral and pulmonary resistance.

RESULTS

The results are presented in Tables I to III. The figures represent mean values, standard deviations, and p value of significance of the change. Significance was considered to be established at the 5 per cent level. It is implicit that "statistically" significant is not necessarily "physiologically" significant. In Group 1 the figures for myocardial metabolism and coronary hemodynamics are shown from 12 patients only.

Group 1: Normal Subjects. General Metabolism (Table I).—The respiratory exchange increased significantly (by 1.2 L. per minute) as did the arterial oxygen content and extraction rate for oxygen. There was a definite decline in the mixed venous and arterial carbon dioxide contents and the difference between these two increased ($p < 0.05$). No change in the pH* was found.

Table II shows the changes in systemic and pulmonary hemodynamics consequent to the injection of aminophylline. The cardiac index tended to fall and, although neither this factor nor the mean systemic arterial pressure was statistically changed, the resultant calculated peripheral resistance was increased ($p < 0.05$). Left ventricular work showed only a trend toward a decrease. The significant fall in right atrial mean pressure from 6.9 to 3.8 ($p < 0.001$) was reflected in a definite fall in right ventricular end-diastolic and pulmonary mean arterial pressures. These changes, with the fall in cardiac output, reduced both

*As measured by Cambridge pH meter.

TABLE I. GENERAL METABOLISM (FIGURES ARE MEANS WITH STANDARD DEVIATIONS)

FACTOR	CONTROL			EXPERIMENTAL			P VALUE* OF CHANGE		
	GROUP 1	GROUP 2	GROUP 3	GROUP 1	GROUP 2	GROUP 3	GROUP 1	GROUP 2	GROUP 3
Respiratory volume (L./min.)	5.0 ± 0.7	7.0 ± 2.2	6.9 ± 3.0	6.2 ± 1.8	7.6 ± 2.2	7.5 ± 2.9	<0.05	<0.1 >0.05	<0.05
Oxygen consumption (ml./min.)	264 ± 31	224 ± 40	225 ± 17	277 ± 40	223 ± 36	239 ± 15	>0.3	>0.9	>0.2
Carbon dioxide product (ml./min.)	209 ± 10	180 ± 34	180 ± 47	224 ± 19	179 ± 31	185 ± 43	>0.3	<0.9	>0.5
Respiratory quotient	0.79 ± 0.05	0.81 ± 0.08	0.88 ± 0.09	0.80 ± 0.12	0.80 ± 0.06	0.86 ± 0.11	>0.7	>0.9	>0.7
Arterial O_2 (vol. per cent)	18.6 ± 0.9	16.6 ± 2.3	16.2 ± 2.4	19.0 ± 1.0	16.5 ± 2.2	15.8 ± 2.6	<0.05	>0.9	>0.1
Mixed venous O_2 (vol. per cent)	15.1 ± 0.9	11.3 ± 2.4	11.2 ± 3.3	14 8 ± 1.0	10.8 ± 2.1	10.2 ± 3.0	>0.1	<0.6	<0.01
ΔArterial-mixed venous O_2 (vol. per cent)	3.6 ± 0.4	5.2 ± 0.8	5.0 ± 1.0	4.2 ± 0.4	5.6 ± 0.3	5.6 ± 1.0	<0.01	<0.1	<0.1
Mixed venous CO_2 (vol. per cent)	50.6 ± 6.0	47.8 ± 3.3	54.3 ± 4.8	48.7 ± 8.0	45.4 ± 4.4	53.3 ± 5.0	<0.01	<0.4	>0.6
Arterial CO_2 (vol. per cent)	48.1 ± 6.0	43.9 ± 4.4	50.5 ± 7	45.7 ± 8.0	41.2 ± 4.3	49.3 ± 8.0	<0.001	<0.2	<0.05
Mixed venous-arterial CO_2 (vol. per cent)	2.5 ± 0.4	3.9 ± 1.0	3.7 ± 1.1	2.9 ± 0.7	4.1 ± 1.8	3.9 ± 1.0	<0.05	<0.5	<0.3
pH mixed venous	7.41 ± 0.03			7.43 ± 0.03			>0.2		
pH arterial	7.42 ± 0.04			7.44 ± 0.03			>0.7		

*Tests the significance of change.

TABLE II. SYSTEMIC AND PULMONARY HEMODYNAMICS (FIGURES ARE MEANS WITH STANDARD DEVIATIONS)

FACTOR	CONTROL			EXPERIMENTAL			p VALUE* OF CHANGE		
	GROUP 1	GROUP 2	GROUP 3	GROUP 1	GROUP 2	GROUP 3	GROUP 1	GROUP 2	GROUP 3
Heart rate per minute	80 ± 9	78 ± 19	84 ± 18	78 ± 9	81 ± 20	86 ± 22	<0.05	>0.2	>0.3
Cardiac index (L./min./M.²)	4.0 ± 0.7	2.7 ± 0.3	2.7 ± 0.9	3.5 ± 0.6	2.5 ± 0.27	2.6 ± 0.9	<0.2	>0.05 <0.1	>0.5
Right atrial pressure (mean, mm. Hg)	6.9 ± 1.4			3.8 ± 0.9			<0.001		
Systemic arterial pressure (mean, mm. Hg)	96 ± 5.0	87 ± 12	93 ± 15	96 ± 8.0	82 ± 10	94 ± 16	>0.9	>0.2	>0.5
Left ventricular work index (Kg./M./min./M.²)	5.1 ± 0.8	3.2 ± 0.18	3.3 ± 0.8	4.6 ± 1.1	2.8 ± 0.4	3.2 ± 0.8	>0.2	<0.05	>0.7
Systemic resistance (dynes. sec. cm.⁻⁵)	1,031 ± 218	1,632 ± 430	1,884 ± 900	1,184 ± 206	1,704 ± 375	1,954 ± 900	<0.05	>0.3	>0.6
Right ventricular end-diastolic pressure (mm. Hg)	8.5 ± 1.8			5.4 ± 1.4			<0.001		
Pulmonary arterial pressure (mean, mm. Hg)	17.0 ± 1.9	36 ± 13	29 ± 20	13.0 ± 1.7	32 ± 13	27 ± 24	<0.001	<0.01	<0.01
Pulmonary capillary pressure (mean, mm. Hg)	11.7 ± 1.5			7.4 ± 2.3			<0.001		
Right ventricular work index (Kg./M./min./M.²)	0.9 ± 0.1	1.3 ± 0.4	1.1 ± 0.8	0.6 ± 0.2	1.1 ± 0.4	1.0 ± 0.8	<0.001	<0.02	<0.3
Total pulmonary resistance (dynes. sec. cm.⁻⁵)	180 ± 41	691 ± 300	663 ± 500	153 ± 28	658 ± 300	569 ± 500	<0.05	>0.4	<0.2
Pulmonary arteriolar resistance (dynes. sec. cm.⁻⁵)	50 ± 13			61 ± 26			>0.2		

*Tests the significance of change.

TABLE III. CORONARY HEMODYNAMICS AND MYOCARDIAL METABOLISM OF OXYGEN AND CARBON DIOXIDE (FIGURES ARE MEANS WITH STANDARD DEVIATIONS)

FACTOR	CONTROL			EXPERIMENTAL			p VALUE* OF CHANGE		
	GROUP 1	GROUP 2	GROUP 3	GROUP 1	GROUP 2	GROUP 3	GROUP 1	GROUP 2	GROUP 3
Coronary blood flow (ml./100 Gm./min.)	79 ± 26	80 ± 35	74 ± 36	69 ± 18	69 ± 23	64 ± 23	<0.01	<0.05	<0.1>0.05
Coronary flow/beat (ml.)	1.01 ± 0.89	0.99 ± 0.25	0.89 ± 0.4	0.89 ± 0.6	0.88 ± 0.23	0.76 ± 0.2	<0.05	>0.1	>0.5
Coronary vascular resistance (units)	1.3 ± 0.3	1.27 ± 1.0	1.50 ± 0.7	1.6 ± 0.3	1.3 ± 1.0	1.56 ± 0.7	<0.02	>0.5	>0.9
Coronary blood flow/ L.V. work ratio	15.3 ± 6.0	24.7 ± 8.0	21.7 ± 6.0	14.3 ± 4.5	24.5 ± 5.0	20.0 ± 5.0	>0.5	>0.9	>0.5
Coronary sinus O_2 content (vol. per cent)	6.5 ± 1.1	3.9 ± 0.3	4.8 ± 1.3	5.3 ± 0.9	3.3 ± 0.8	3.9 ± 0.6	<0.01	<0.01	>0.4
ΔArteriol-coronary sinus O_2 (vol. per cent)	12.1 ± 1.0	12.6 ± 1.6	11.2 ± 2.0	13.8 ± 1.2	13.4 ± 1.6	12.1 ± 2.5	<0.01	<0.01	<0.05
Coronary sinus CO_2 content (vol. per cent)	58.3 ± 1.3	53.4 ± 2.9	59.1 ± 1.0	56.8 ± 2.7	51.7 ± 2.8	58.0 ± 7.0	<0.02	<0.05	<0.1>0.05
ΔCoronary sinus arterial CO_2 (vol. per cent)	9.9 ± 1.2	9.8 ± 1.5	8.4 ± 0.9	10.6 ± 1.2	10.5 ± 2.2	8.8 ± 1.3	>0.2	>0.1	>0.2
Cardiac respiratory quotient	0.82 ± 0.15	0.78 ± 0.04	0.76 ± 0.06	0.77 ± 0.07	0.78 ± 0.08	0.74 ± 0.06	>0.3	>0.9	>0.1
Cardiac metabolic rate O_2 (ml./100 Gm./min.)	9.5 ± 2.7	9.8 ± 3.8	8.0 ± 2.6	9.4 ± 2.0	9.1 ± 2.7	7.8 ± 3.0	>0.9	>0.6	>0.8
Cardiac metabolic rate CO_2 (ml./100 Gm./min.)	7.7 ± 2.5	7.6 ± 3.1	6.1 ± 2.4	7.1 ± 1.1	7.0 ± 1.9	6.4 ± 2.6	>0.7	>0.3	>0.1
Index of efficiency (L. V. work ÷ CMR O_2)	0.61 ± 0.21	0.35 ± 0.1	0.45 ± 0.1	0.54 ± 0.12	0.32 ± 0.06	0.44 ± 0.1	>0.3	>0.5	>0.6

*Tests the significance of change.

right ventricular work ($p < 0.001$) and total pulmonary resistance. The fall in pulmonary "capillary" pressure, although significant, failed to cause a definite change in calculated pulmonary arteriolar resistance.

The results concerning coronary hemodynamics and myocardial metabolism for oxygen and carbon dioxide are presented in Table III. The coronary blood flow fell by 10 ml. per 100 Gm. per minute ($p < 0.01$) and, in the face of any real change in heart rate, the coronary flow per beat was reduced. As the systemic mean pressure was unchanged (Table II), the calculated coronary resistance increased ($p < 0.02$).

The coronary sinus oxygen content fell ($p < 0.01$), and the difference between this and the arterial oxygen content increased significantly. This increase, when multiplied by the lower coronary blood flow, kept the cardiac metabolic rate for oxygen at control values. Similar observations for the myocardial carbon dioxide metabolism were present. No changes occurred in the cardiac respiratory quotient, index of efficiency (left ventricular work ÷ CMR O_2), or ratio of coronary blood flow to left ventricular work.

Group 2: Patients With Mitral Stenosis.—In these patients the results expressed in Tables I and II were similar in trend to those recorded for Group 1. Significant results were, however, achieved only in reductions in mean pulmonary arterial pressure and right and left ventricular work indices.

As far as coronary hemodynamics were concerned, a significant decrease in coronary blood flow occurred; resistance was not significantly increased. (Table III.) The oxygen and carbon dioxide contents of the coronary sinus blood decreased significantly; an increase ($p < 0.01$) in arterial/coronary sinus oxygen difference was found. As in Group 1, the cardiac metabolic rates for oxygen and carbon dioxide remained virtually unchanged. Values for index of efficiency, coronary blood flow/left ventricular work ratio, and cardiac respiratory quotient, as in Group 1, showed no significant change.

Group 3: 6 Patients With Cor Pulmonale, 2 With Myxedema.—In these patients fewer significant changes were observed. As in the other two groups, the minute volume of respiration increased significantly, and there was a reduction in arterial and mixed venous carbon dioxide levels. There was, however, a definite ($p < 0.05$) increase in myocardial arteriovenous oxygen difference. This again kept the cardiac metabolic rate for oxygen at control values. Similar statements apply to myocardial carbon dioxide exchange.

DISCUSSION

The changes recorded in general metabolism and hemodynamics agree broadly with reports already referred to. As seems reasonable, the results noted for Group 1 (normal subjects) show more consistency. In the present study, cardiac output was statistically unchanged, perhaps related to the time at which this factor was measured since Howarth and associates[6] observed an early (within 5 minutes) rise, followed, as here, by a decrease. It was not possible to confirm the fall in systemic blood pressure noted by Storstein and his co-workers[8] perhaps because they used an auscultatory method of estimating mean blood pressure. An increase in calculated peripheral resistance was found in Group 1.

As has been previously found,[6, 8] the mean pressure in the right atrium fell significantly, and, with reference to Group 1, this decrease was paralleled by a similar fall (about 4 mm. Hg) in right ventricular end-diastolic, mean pulmonary arterial, and pulmonary capillary pressures. Although total pulmonary resistance fell significantly, the unchanged pulmonary arteriolar pressure suggests that this drug is not, at least in normal subjects, a true pulmonary vasodilator. Werkö and Lagerlöf[21] observed (in patients who were not decompensated), that the central (heart and lung) volumes were unchanged. In this light, the fall in mean right atrial pressure suggests a degree of "pooling" in the periphery. This decrease in atrial pressure is perhaps a main cause of the decrease in pulmonary pressures that have been observed.

The changes discussed above, together with those in ventilation, suggest that the drug was exerting an effect upon the body, including the myocardium at the time of study. The timing of the coronary flow studies was identical in the animal[4] and in a study of cerebral metabolism after aminophylline in man.[22] These facts with the evidence for the duration of effect suggested by Werkö and Lagerlöf[21] suggest that the study should be valid in relation to any thesis concerning an effect upon the myocardium.

In two groups studied (normal individuals and patients with mitral stenosis) the coronary blood flow fell significantly; the trend was maintained in the third group. The reason for the fall is not completely understood, but cardiac rate, the change of which may influence coronary blood flow,[23, 24] was unaffected and may be discounted as a factor. The decrease in left ventricular work (significant only in Group 2) may have contributed to the decline in coronary blood flow.[25] The decrease in coronary sinus oxygen level could be interpreted as a direct effect of the drug upon the myocardium, but the maintenance of the cardiac metabolic rate for oxygen at control values echoes Barcroft's "dictum" and suggests that the increased extraction is associated with the decreased flow. The changes outlined above for the heart compare closely with those recorded for the cerebral circulation by Wechsler and associates[22] who described an increase in cerebral vascular resistance and widened cerebral oxygen extraction; they implied that cerebral anoxia was present. Certainly there is no evidence of any beneficial effect upon the cardiac circulation in the normal and diseased persons presented here. There is no evidence from this study as to how long the effects discussed may persist, nor any explanation as to why the apparent vasodilatory effect noted in the nonintact animals could not be reproduced. The results presented would tend to confirm the lack of beneficial effect noted in clinical studies of the effect of aminophylline in angina.[13, 14]

SUMMARY

A study has been made of the effects of aminophylline upon the normal and diseased heart. In normal persons and patients with mitral stenosis, coronary blood flow decreased, with increased myocardial oxygen extraction. The cardiac metabolic rate for oxygen was maintained. No evidence of a coronary vasodilatory action was found.

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STUDIES OF FREE COLLAPSE IN THE INTACT HUMAN LUNG

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THE lungs empty purely from the forces of their elastic properties when no external pressure is applied. Free collapse of the lungs may be said to exist when the intrathoracic pressure approximates atmospheric pressure provided that the upper air passages are widely patent. Studies of free collapse are of considerable interest because they permit an assessment of the volume elastic and the flow resistive properties of the lungs. Such studies have not been reported previously for the human lung.

The ordinary pneumotachograph is not particularly suited for this type of study inasmuch as the volume data thus obtained depend upon integration of the volume flow rate curve. Any error in the flow data would appear, therefore, in the volume measurement. The development of a servomotor spirometer* which affords negligible resistance to air flow and which simultaneously, but independently, measures volume and volume flow rate made the present study possible.

SUBJECTS AND METHODS

Eight healthy house officers and medical students served as subjects. No studies were performed during the period of a respiratory infection. The physical characteristics of the subjects and the results of lung function studies are shown in Table I. The mean age was 25 years.

Routine spirometric and residual volume determinations were performed with the subjects sitting upright. An open circuit nitrogen dilution technique was employed for the residual volume measurements.[1] All gas volumes have been corrected to 37° C. saturated with water vapor. The intraesophageal balloon utilized in the present studies has known frequency response characteristics.[2] The pressure volume diagram of the lungs was determined during expiration by the method of Stead, Fry, and Ebert.[3] Resistance to air flow was determined at the level of pulmonary mid-capacity during expiration by the method of Fry and co-workers.[4]

A special servomotor spirometer was used to record simultaneously volume and volume flow rate during free collapse. Following maximal inspiration, the subject pressed his face against a rubber tubing two inches in internal diameter which led to the special spirometer. With the mouth open widely, intrathoracic pressure was adjusted to an atmospheric level by the sudden relaxation of the respiratory muscles. This maneuver simulated a sigh.

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*Model 150, Servo-Spirometer, manufactured by the Custom Engineering & Development Company, St. Louis 9, Mo.

TABLE I. PHYSICAL CHARACTERISTICS AND LUNG FUNCTION STUDIES IN EIGHT SUBJECTS

SUBJECT	AGE (YR.)	HEIGHT (CM.)	WEIGHT (KG.)	BODY SURFACE AREA (SQ. M.)	TOTAL LUNG CAPACITY (L.)	VITAL CAPACITY (L.)	RATIO RV/TLC* (%)	MIXING INDEX† (%)	MAXIMUM BREATHING CAPACITY (L./MIN.)	COMPLIANCE (L./CM. H_2O)	K_1 (CM. H_2O/ L./SEC.)	K_2 (CM. H_2O/ L.^{2}/SEC.^{2})
S. L.	26	185	68	1.90	8.19	6.38	22.1	0.50	154	0.333	0.70	0.46
J. B.	24	180	78	1.98	7.61	6.09	20.0	0.34	154	0.235	1.05	0.31
P. R.	28	168	70	1.80	5.75	4.60	20.0	0.55	166	0.167	1.52	0.44
J. H.	24	176	91	2.08	7.11	5.37	24.5	0.51	182	0.270	0.95	0.55
D. P.	26	177	74	1.91	5.64	4.75	15.8	0.40	131	0.189	1.22	0.83
G. M.	27	175	72	1.87	6.48	5.25	19.0	0.73	142	0.281	1.06	0.57
M. C.	24	170	81	1.93	6.48	5.32	17.9	0.37	205	0.213	1.38	0.36
H. R.	25	185	89	2.14	7.19	6.03	16.1	0.42	139	0.278	1.91	0.45
Mean	25	177	78	1.95	6.81	5.47	19.4	0.48	159	0.246	1.22	0.50
Standard error	1.5	6	8	0.11	0.89	0.64	2.9	0.12	24	0.055	0.38	0.16

*Ratio of residual volume to total lung capacity.
†Nitrogen concentration in alveolar gas after 7 minutes of breathing pure oxygen.

Pressure was recorded differentially with a strain gauge transducer from the intraesophageal balloon and from the mouth connecting tube. Records were obtained photographically with a multichannel electronic recorder. All records accepted for analysis showed a transpulmonary pressure of less than two centimeters of water during the period of free collapse. Fig. 1 is a reproduction of one of the crude records. There was close agreement between the attainment of an atmospheric level of intrathoracic pressure and the occurrence of the maximal volume flow rate. Accordingly, the time of maximal flow was considered as the onset of free collapse. The volume of lung inflation at this time will be referred to as volume zero, V_0. The terminal portion of the vital capacity, which is not shown in the figure, was forcibly expelled in order to be certain that the size of the measured breath corresponded with the subject's known vital capacity.

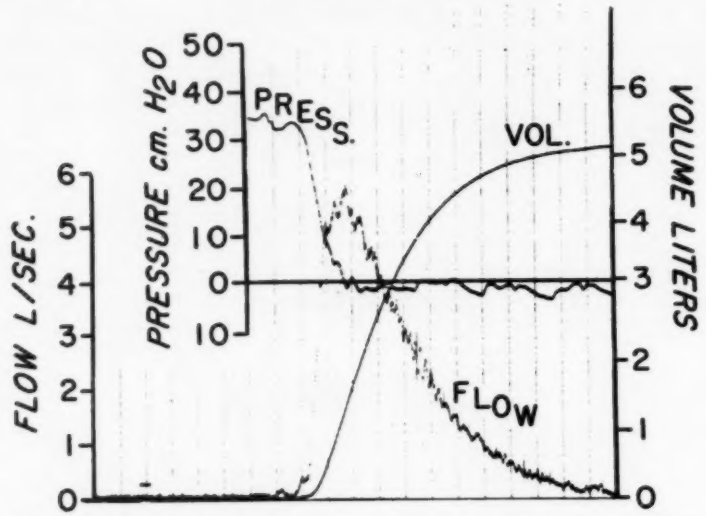

Fig. 1.—Crude record of free collapse. Pressure is negative above the horizontal line marked zero. The faint vertical lines represent time in 0.2 second intervals.

The pressure in the chamber of the servomotor spirometer and in the short connector tubing was found to deviate from atmospheric pressure by less than 0.5 cm. of water under the most extreme conditions of flow. This permitted the conclusion that intraoral pressure was approximately atmospheric during free collapse of the lungs. Furthermore, this indicated that the measured transpulmonary pressure was equal to intrathoracic pressure.

Intraesophageal pressure was used as a measure of intrathoracic pressure. Comparison of intraesophageal pressure with intrapleural pressure would indicate that the intraesophageal pressure is slightly more positive.[5] This means that free collapse occurred at levels of intrapleural pressure which were slightly below atmospheric. A correction has not been applied, however, since the exact amount of error is somewhat obscure and since there is some question as to whether intrapleural pressure is identical in all parts of the chest. Because of the inaccuracies already described, and because the intraesophageal pressure fluctuates with each cardiac cycle, only those levels of lung inflation have been included where the force of the elastic properties is large, that is, above pulmonary mid-capacity. This tended to minimize the effect of deviations from atmospheric levels of intrathoracic pressure during free collapse of the lungs.

The validity of the present study rests on the reliability of the servomotor spirometer. Hence, this spirometer was calibrated by using a standard 9 L. spirometer (Table II). It

was further determined that the potentiometer which measures volume and the tachometer which measures volume flow rate in the servomotor spirometer responded in a linear fashion over the ranges employed in this study. Although the calibration of the manufacturer indicated satisfactory frequency response characteristics, it was established that the instrument was capable of a change in volume flow rate from 0 to 4 L. per second in less than 0.05 second.

TABLE II. CALIBRATION SUMMARY

	STANDARD SPIROMETER VOLUME*	SERVOMOTOR SPIROMETER VOLUME†	SERVOMOTOR SPIROMETER FLOW
Mean factor‡	0.977	0.941	0.928
Standard deviation	0.025	0.013	0.027
Number of observations	45	82	85

*The standard 9 L. spirometer was calibrated with distilled water. The weight of the distilled water was converted into volume using the table on page 1954 of the *Handbook of Chemistry and Physics*, ed. 35, Cleveland, 1953, Chemical Rubber Publishing Company.

†The servomotor spirometer was calibrated with the standard 9 L. spirometer.

‡This factor divided into the spirometer scale reading will yield the true volume.

Mathematical Analysis.— An analysis of free collapse requires several basic considerations. For simplicity, it is necessary to assume that the pressure volume diagram of the lungs is linear. This assumption is approximately true about the level of pulmonary mid-capacity. It is not true, however, at either extremely high or extremely low levels of lung inflation. An accepted expression for this relationship is:

$$P = KV \tag{1}$$

where P is pressure in centimeters of water, K is a constant, and V is volume in liters.

An accepted expression for the resistance to air flow at any given level of lung inflation is:

$$P = K_1\dot{V} + K_2\dot{V}^2 \tag{2}$$

where P is pressure in centimeters of water, $\dot{V}$ is the volume flow rate in liters per second, and K_1 and K_2 are the flow resistive constants. This equation was proposed by Rohrer in 1915.[6] It has been established that the resistance to expiratory air flow is decreased 1 L. above pulmonary mid-capacity as compared to mid-capacity.[4] This means that the values for K_1 and K_2 are smaller at the higher levels of lung inflation.

An equation for the dynamic relationships of these factors under the conditions of free collapse is:

$$KV + K_1\dot{V} + K_2\dot{V}^2 = 0 \tag{3}$$

where the symbols are the same as above. The assumptions are made that inertia is negligible, that K_1 and K_2 participate at all levels of lung inflation as determined at pulmonary mid-capacity, and that tissue frictional resistance is expressed by the term $K_1\dot{V}$ or $K_2\dot{V}^2$. From this equation it is apparent that free collapse is determined by the volume elastic and the flow resistive characteristics of the system.

In order to predict the volume time course of free collapse from all 3 measured constants, an analytical solution for this equation was obtained. Solved for time in seconds this solution is:

$$t = \frac{K_1}{2K}\left\{\log_e \frac{[V_o]\left[\sqrt{\left(\frac{K_1}{2K_2}\right)^2 - \frac{K}{K_2}V} + \frac{K_1}{2K_2}\right]\left[\sqrt{\left(\frac{K_1}{2K_2}\right)^2 - \frac{K}{K_2}V_o} - \frac{K_1}{2K_2}\right]}{[V]\left[\sqrt{\left(\frac{K_1}{2K_2}\right)^2 - \frac{K}{K_2}V} - \frac{K_1}{2K_2}\right]\left[\sqrt{\left(\frac{K_1}{2K_2}\right)^2 - \frac{K}{K_2}V_o} + \frac{K_1}{2K_2}\right]}\right\} - \frac{2K_2}{K}\left\{\sqrt{\left(\frac{K_1}{2K_2}\right)^2 - \frac{K}{K_2}V} - \sqrt{\left(\frac{K_1}{2K_2}\right)^2 - \frac{K}{K_2}V_o}\right\} \tag{4}$$

V_o was defined above. It should be pointed out that in the observed free collapse curves, the maximal volume flow rate occurred approximately 1 L. below the level of maximal lung inflation. Hence, V_o in the predicted curves was 1 L. below total lung inflation.

In order to predict the volume time course for an assumed pure laminar flow, an analytic solution was also obtained for equation 3 with the assumption that the term $K_2\dot{V}^2$ was negligible. This solution is:

$$V = V_o e^{-\frac{K}{K_1}t} \tag{5}$$

In the mathematical analysis, the additional assumption is required that the individual products of resistance and compliance for the smallest functional units of the lungs are equal throughout. It is probable that this assumption is valid in healthy young subjects.

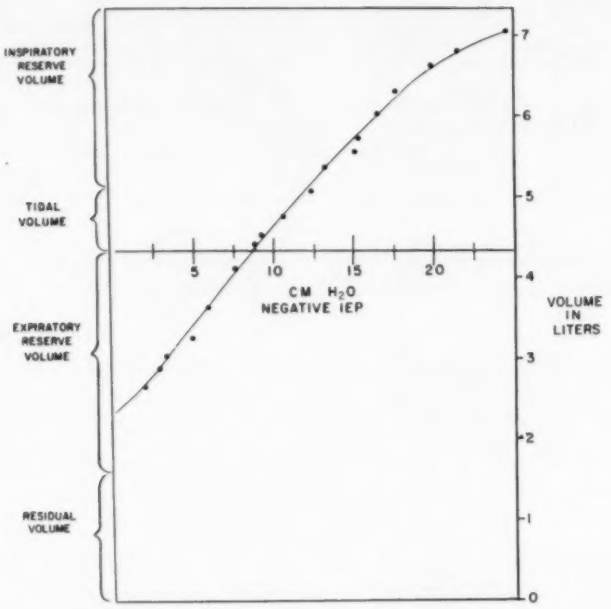

Fig. 2.—Static pressure volume diagram of the lungs.

RESULTS

The mean static pulmonary compliance measured during expiration was 0.25 ± 0.05 L. per centimeter of water. Fig. 2 is a representative pressure volume diagram of the lungs from one of the subjects. This individual was re-examined after an interval of 20 months and found to have a pulmonary compliance within 0.01 L. per cm. H_2O of the original value. Moreover, the level of pulmonary mid-capacity as measured both on the spirometer tracing and by the intraesophageal pressure was closely similar in the two studies.

The nonlinear portion of the pressure volume diagram, as seen in Fig. 2, usually extended approximately 1 L. below the level of maximal lung inflation.

In two of the present study subjects, however, there was a marked curvilinear relationship of pressure to volume which extended 2 L. below maximal lung inflation.

Measured at the level of pulmonary mid-capacity during expiration, the mean linear flow resistive constant (K_1) was 1.2 ± 0.4 cm. H_2O/L./sec., and the mean quadratic flow resistive constant (K_2) was 0.5 ± 0.2 cm. H_2O/L.^{2}/sec.^{2} It was established in two subjects that these measurements were also reproducible. Care was taken in this procedure to avoid occlusion of the teeth as this will markedly increase the resistance to air flow. It should be pointed out that the small quantity of pressure required for frictional resistance in the tissue is included in these constants.

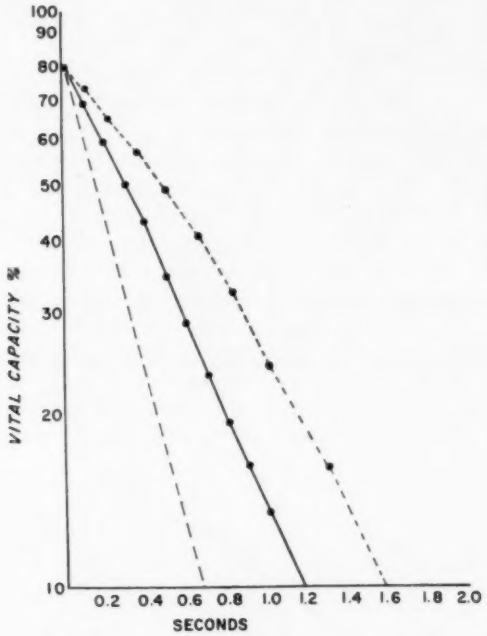

Fig. 3.—Volume time course of free collapse. The solid center line is the observed free collapse. The interrupted line on the left is the calculated course for an assumed pure laminar flow. The dotted line on the right is the calculated course for combined laminar and turbulent flow.

A representative free collapse curve is presented in Fig. 3. The curve of the logarithm of volume plotted against time is approximately linear. The calculated curve for the assumption that the flow is entirely laminar follows a more rapid volume time course than the observed free collapse curve. The calculated curve for the assumption that flow is both laminar and turbulent follows a less rapid volume time course than the observed curve. While some of the 45 curves closely approximated the pure laminar flow prediction, others

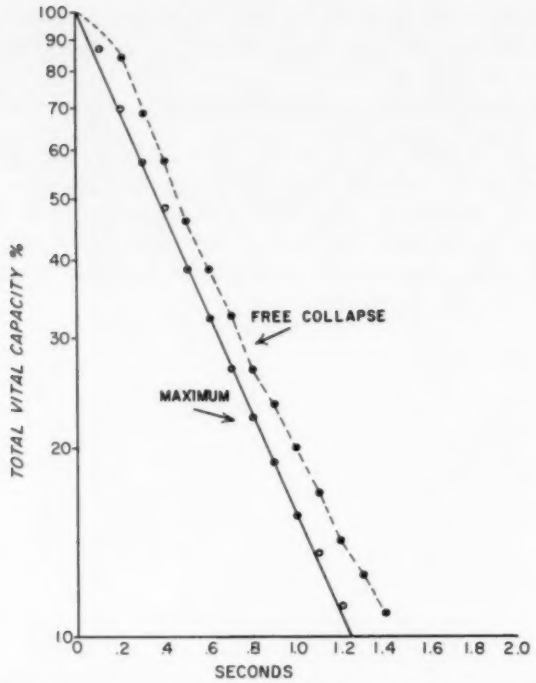

Fig. 4.—Comparison of a maximally rapid vital capacity with free collapse of the lungs.

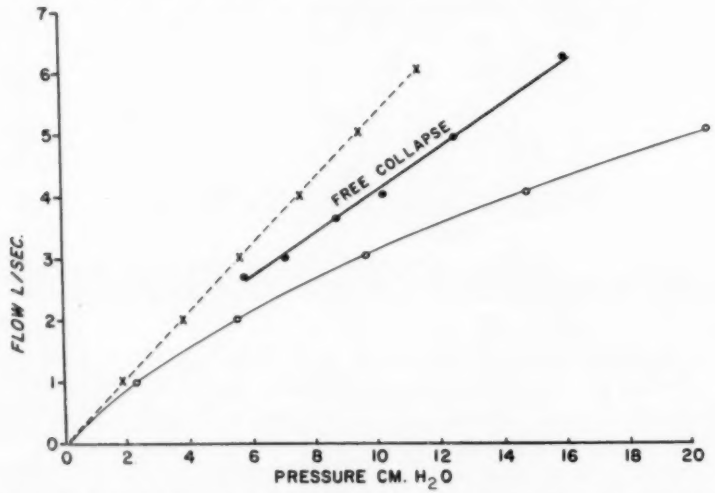

Fig. 5.—Free collapse of the lungs. The volume flow rate on the ordinate is plotted against the pressure of the elastic properties of the lungs on the abscissa. The solid center line is the observed free collapse. The broken line (above) is the calculated relationship for an assumed laminar flow. The curved solid line (below) is the calculated relationship for an assumed laminar and turbulent flow.

more closely approximated the combined laminar plus turbulent flow prediction. It is striking that in every instance the observed free collapse was intermediate between the two predicted curves.

A representative curve of free collapse was compared with a maximally rapid expulsion of the vital capacity in each subject. There was a longer lag prior to the onset of rapid air flow in the case of free collapse as compared to the maximal effort. As shown in Fig. 4 both of these curves were roughly exponential during the period of rapid air flow. Consequently, the best linear fit during this period was obtained by inspection of the points on semi-logarithmic paper. The slope of this line may be expressed conveniently as the half time. In 8 subjects, the mean half time was 0.34 second for maximal effort, and 0.41 second for free collapse. This expression eliminates the lag period, which is not of interest here. This comparison was also made with the measurement of mid-expiratory flow by the technique of Leuallen and Fowler.[7] The mean mid-expiratory flow was 4.96 L. per second for the maximal effort, and 3.91 L. per second during free collapse.

Free collapse may be viewed in another manner as illustrated in Fig. 5. This shows pressure plotted against volume flow rate. The volume flow rate was measured on the free collapse record at intervals of 0.1 second. The pressure at each interval was obtained by referring the measured volume below the total lung capacity to the pressure volume diagram of the lungs for the individual. The values are plotted only to the level of pulmonary mid-capacity because the most reliable data are obtained above this level of lung inflation. Once again, the observed free collapse was situated between the laminar flow prediction and the prediction for combined laminar plus turbulent flow. In Fig. 5, any straight line which passes through the point of origin of the scale reflects a constant ratio of pressure to flow. There was a tendency for a linear extension of the observed free collapse lines to pass near the point of origin.

TABLE III. RATIO OF PRESSURE TO FLOW DURING FREE COLLAPSE OF THE LUNGS

SUBJECT	MEAN RATIO PRESSURE/FLOW (CM. H_2O/L./SEC.)	RANGE (CM. H_2O/L./SEC.)	STANDARD ERROR (CM. H_2O/L./SEC.)	NUMBER OF CURVES STUDIED
S. L.	2.32	2.04-2.51	0.18	6
J. B.	1.91	1.59-2.49	0.35	6
P. R.	1.88	1.77-2.05	---	4
J. H.	1.98	1.64-2.50	0.31	8
D. P.	2.05	1.92-2.26	0.13	6
G. M.	2.10	1.80-2.36	---	3
M. C.	2.30	1.89-2.72	0.28	6
H. R.	2.54	2.36-2.69	0.13	6
Mean	2.14	1.59-2.72	0.31	

If the bronchi were rigid and if air flow were entirely laminar, the ratio of pressure to flow (that is, the resistance) would remain constant at various flow rates. Inasmuch as there is turbulent as well as laminar flow in the bronchial tree, the ratio of pressure to flow would be expected to increase as the flow increases. To test this hypothesis, the ratio of pressure to flow was measured during free collapse at 197 points on 45 curves. The level of lung inflation was pulmonary mid-capacity or greater at each point. The mean value

was 2.1 ± 0.3 cm. H_2O per L. per second. The data obtained from the individual subjects are shown in Table III. There was no significant difference in the resistance to air flow at various rates of flow between 2 and 6 L. per second. This finding is of interest because previous measurements of the resistance to air flow have been made at much lower rates of flow. This observation seems to be explained by the elastic properties of the conducting airways. It is, of course, well known that the bronchi have larger diameters at high levels of lung inflation.[8] Obviously, an increase in the diameter of the bronchi will result in a decrease of the resistance to air flow.

DISCUSSION

Free collapse was defined as that condition which results when the lungs are permitted to empty from the forces of their elastic properties alone. It is apparent that when the elastic forces of the lungs are increased (a decreased pulmonary compliance), the volume time course of free collapse will be shortened. When the resistance to air flow is increased, the time required for collapse will be lengthened. Opposite changes in compliance and resistance will reverse these effects.

The present study was done to determine whether or not the lungs, during free collapse, would behave in a manner predictable on the basis of information obtained from a static compliance curve and a pressure flow curve. It was not expected that free collapse would follow perfectly either of the predicted volume time courses. The calculated time course based on the assumption of pure laminar flow depends entirely on the ratio of the linear flow resistive constant, K_1, to the pulmonary elastance, K. Since K_1 comprises only a part of the total resistance to air flow, this prediction follows too rapid a volume time course. The calculated time course based on the assumption of both laminar and turbulent flow depends on the participation of both flow resistive constants, K_1 and K_2, as measured at the level of pulmonary mid-capacity. Inasmuch as these values tend to be smaller at levels of lung inflation above pulmonary mid-capacity, the calculated volume time course of combined laminar and turbulent flow is unduly prolonged. A more accurate prediction of the volume time course of free collapse would require a knowledge of the change which occurs in the flow constants, K_1 and K_2, at various levels of lung inflation. Although this information is not available, the present results indicate that the static method employed for the measurement of pulmonary compliance and the interrupter method used to measure the resistance to air flow are reasonably valid in the dynamic state of free collapse.

The volume time course of free collapse was compared to the course of the maximally rapid vital capacity. The transpulmonary gauge pressures were markedly different being approximately zero during free collapse, and between 60 and 80 cm. of water with maximal effort. This means that the intra-alveolar pressure was from 3 to 10 or more times as large during the maximal effort as during free collapse. In spite of this, the mean half time during the period of rapid air flow was not markedly different between maximal effort and free collapse. Fry[9] and Hyatt[10] have recently pointed out that with levels of lung

inflation in the lower one-half of the vital capacity, the maximal obtainable expiratory flow does not occur with maximal effort, but at airway pressure gradients in the order of 0 to 25 cm. H_2O. The present data on free collapse were obtained only at the level of pulmonary mid-capacity or higher, and hence are not strictly comparable to the data of Fry and Hyatt. The implication from both of these studies, however, is that the physical properties of the lungs and lower airways are dominant in the control of air flow during expiration.

Comroe and associates[11] suggested that curves of passive expiration could be used to assess rapidly the mechanical properties of the lungs and chest. Brody[12] and Ebert and Haddy[13] studied passive expiration in dogs. In both of these studies the initial level of lung inflation was obtained by opening the trachea to the atmosphere. It is uncertain whether anesthetized or paralyzed animals always return to the same level of lung inflation upon opening the trachea to the atmosphere. In fact, it seems probable that they do not. Another difficulty with interpretation of the data of Ebert and Haddy is that the administration of curare results in a transient decrease in pulmonary compliance.[14] Brody presented a mathematical analysis similar to but less complete than the present one. Both Brody and Ebert and Haddy concluded that inertia was negligible.

SUMMARY

1. Free collapse of the lungs was studied in healthy young men by the rapid voluntary adjustment of intrathoracic pressure to an atmospheric level. A pressure constant spirometer was used to measure volume and volume flow rate.

2. A mathematical analysis of the mechanics of free collapse was presented.

3. An analysis of 45 free collapse curves revealed, within limits, that the behavior of the lungs could be predicted from static measurements of pulmonary compliance and interrupter measurements of the resistance to air flow.

4. The mean ratio of pressure to expiratory air flow during free collapse was 2.1 cm. of water per liter per second when measured at flow rates between 2 and 6 L. per second.

5. There was no increase in this ratio per unit of flow at rapid volume flow rates. This finding was attributed to the elastic properties of the bronchi and conducting airways.

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LABORATORY METHODS

EVALUATION OF AN ISOLATION AND MAINTENANCE MEDIUM FOR ACTINOMYCES SPECIES AND RELATED ORGANISMS

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DURHAM, N. C.

IN HIS review on the parasitic actinomycetes, Rosebury[12] emphasized the difficulties involved in isolation and maintenance of pure cultures of *Actinomyces israeli*. This difficulty, whereby many laboratories are unable to grow and maintain cultures of the anaerobic actinomycetes, was re-emphasized more recently by the work of Erikson and Porteous.[2]

Recently Howell and Pine[5] described a synthetic medium which supported excellent growth of many strains of Actinomyces isolated from man. Although the casitone medium was satisfactory as a research tool many requests have been received for a more practical medium which would be cheaper, more easily prepared, and more suitable for general laboratory use. Therefore, it is the purpose of this paper to describe a modified casitone medium which has been used for the successful isolation, cultivation, or maintenance of 18 strains of *Actinomyces israelii*, and 11 strains of *A. bovis*.* The medium has also been found satisfactory for the growth, maintenance, and/or isolation of strains of *Corynebacterium acnes*, *C. liquefaciens*, *Lactobacillus bifidus*, and *Leptotrichia buccalis*. The medium has been tested in several different laboratories and found to be superior to the media which they had been using for the growth of Actinomyces.

MATERIALS AND METHODS

Cultures Used.—All the strains of Actinomyces and *Lactobacillus bifidus* 308 described previously[5, 8] were used in this study. Strains 534, 895, and 699 of *A. israelii* were isolated from cases of human actinomycosis at Duke University Hospital. *A. bovis* strains A1, A8, A9, A10, A11, and A12 were obtained from Dr. William Kaplan and Dr. Libero Ajello, Communicable Disease Center, Chamblee, Ga.; *A. bovis* strain A 601 was received from Dr. Ester Meyer, Department of Bacteriology, University of Illinois. *A. bovis* strains P1, P2, P3, and P4 were isolated at Duke University from typical cases of lumpy jaw of cattle. Strains 552, 585, and 611 of *Leptotrichia buccalis*[1] were obtained from Dr. Arden Howell, Jr., National Institute of Dental Research, Bethesda, Md. *Corynebacterium acnes* and *C. liquefaciens* were American Type Culture Collection cultures Nos. 6919 and 8374, respectively.

From the Department of Microbiology, Duke University Medical School, Durham, N. C.

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*The species designation "israelii" is used here to designate those cultures isolated from man, the term "bovis" is restricted to those strains isolated from bovine cases of lumpy jaw.

Media.—The general media used were nutrient agar containing starch (Garrod's medium)* with and without 10 per cent whole blood, and brain heart infusion (BHI) agar (Difco) with and without 10 per cent whole blood. The incubation temperature for all cultures was 37° C.

Anaerobiosis.—All slants, agar deeps, and tubes of broth were incubated under a saturated pyrogallol and 10 per cent sodium carbonate seal. In practice an absorbent cotton plug was pushed down on top of the cotton stopper of the test tube. Five drops of each reagent was added, and the tube was sealed with a rubber stopper. All cultures were maintained under such seals except cultures of Leptotrichia which were grown aerobically. Agar plates were incubated in an anaerobic jar which was evacuated and flushed 3 times with 95 per cent nitrogen and 5 per cent carbon dioxide.

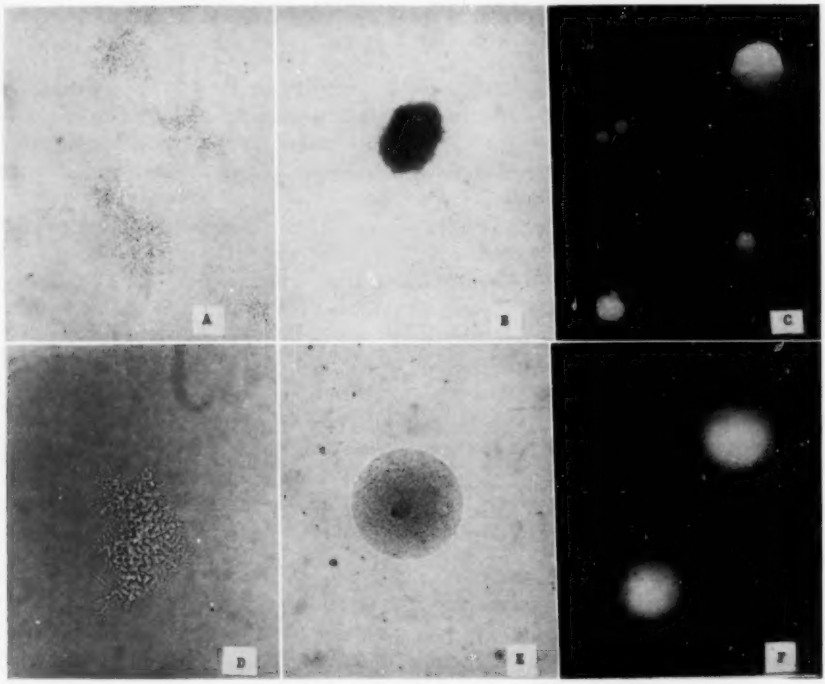

Fig. 1.—Development of colonies of *Actinomyces israelii* and *Actinomyces bovis* on stock Actinomyces medium. *A*, *A. israelii*, young colony under coverslip. (Magnification ×1,000; reduced ⅖.) *B*, *A. israelii*, small colony. (Magnification ×124; reduced ⅖.) *C*, *A. israelii*, large colony. (Magnification ×14; reduced ⅖.) *D*, *A. bovis*, young colony under coverslip. (Magnification ×1,000; reduced ⅖.) *E*, *A. bovis*, small colony. (Magnification ×124; reduced ⅖.) *F*, *A. bovis*, large colony. (Magnification ×14, reduced ⅖.)

Evaluation of Growth.—Growth on the various media tested was evaluated turbidimetrically as previously described,[8] by colony counts, or by subjective scoring with macroscopic observation. The ability of the medium to support successive growth of single microcolonies such as those depicted in Fig. 1, *A* and *D*, was also used as a criterion for evaluation of the medium.

*We are grateful to Dr. Arden Howell for the formula of this medium which is: beef extract 3.0 Gm., peptone 10.0 Gm., NaCl 5 Gm., soluble starch 1.0 Gm., and 20 Gm. agar per liter of medium.

RESULTS

Development of the Stock Actinomyces Medium.—Table I gives the formula for the stock Actinomyces medium as it has been used for approximately two years. During this period all the cultures of *A. israelii, A. bovis, C. acnes, C. liquefaciens, L. bifidus,* and *L. buccalis* described in Materials and Methods were successfully maintained without change in characteristics, cultural or microscopic, as described for each species. Fig. 1 shows the morphologic appearance of the Actinomyces species on this medium. All cultures with the exception of the Leptotrichia were grown in 0.7 per cent agar stabs with the

TABLE I. ACTINOMYCES STOCK MEDIUM

	PER LITER
PART I	
1. Actinomyces salts (4× strength)	250 ml.
60 Gm. KH_2PO_4	
4.0 Gm. $(NH_4)_2SO_4$	
0.8 Gm. $MgSO_4 \cdot 7H_2O$	
0.08 Gm. $CaCl_2$	
2. Casitone (Difco)	4 Gm.
3. Cysteine HCl	1 Gm.
4. Minor element solution	10 ml.
$FeSO_4 \cdot 7H_2O$ 2.0 Gm./500 ml.	
$MnSO_4 \cdot 2H_2O$ 0.075 Gm./500 ml.	
$NaMoO_4 \cdot 2H_2O$ 0.075 Gm./500 ml.	
Add 1 ml. of concentrated HCl to 100 ml. of distilled water. Then add minor elements separately and make to 500 ml.	
5. Purines and pyrimidines	10 ml.
Adenine sulfate 200 mg./100 ml.	
Guanine HCl·$2H_2O$ 200 mg./100 ml.	
Uracil 200 mg./100 ml.	
Xanthine 200 mg./100 ml.	
Thymine 200 mg./100 ml.	
6. Yeast extract	1 Gm.
7. Coenzyme A: 5 mg./50 ml. distilled H_2O	1 ml.
8. Vitamin solution	10 ml.
Thiamine HCl 20 mg./100 ml.	
Pyridoxine HCl 10 mg./100 ml.	
Pyridoxamine 2HCl 5 mg./100 ml.	
Pyridoxal HCl 5 mg./100 ml.	
Ca-pantothenate 20 mg./100 ml.	
Riboflavin 20 mg./100 ml.	
Nicotinic acid 10 mg./100 ml.	
Nicotinamide 10 mg./100 ml.	
p-Aminobenzoic acid 1.0 mg./100 ml.	
Biotin 0.5 mg./100 ml.	
Folic acid 0.5 mg./100 ml.	
Make to pH 6.5 with 20 per cent KOH and dilute to 500 ml.	
PART II	
H_2O 500 ml.	
Glucose 5.0 Gm.	
Agar (regular) 7 Gm. for stock stabs	
1.0 Gm. stock Bakers' purified starch	
Oleic acid 1 mg.	
Prepare by suspending the starch in approximately 50 ml. cold water. This suspension is then poured into 450 ml. boiling water and the other components are then added	
Autoclave Part I and Part II and mix while hot for final volume of 1 liter. Dispense and use with pyrogallol-sodium carbonate seal	

pyrogallol-sodium carbonate seal until heavy growth was obtained and were then stored at 5° C. Transfers were made monthly. If growth of the Actinomyces is not allowed to progress too far* at 37° C., the cultures may be stored at 5° C. for periods up to 6 months without loss of viability. All cultures of the Leptotrichia were maintained on 1.5 per cent agar slants incubated aerobically. BHI, Garrod's medium, thioglycollate broth, and nutrient agar do not support satisfactory growth of *L. buccalis*. Growth of *C. acnes* on these media was inferior to that on stock actinomyces medium. In a period greater than two years, our culture of *L. bifidus* has not lost its bifid character although branched rods are more prevalent in the medium of Norris and associates.[7]

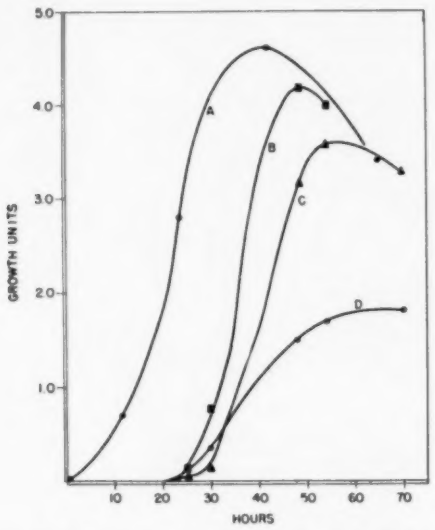

Fig. 2.—Substances influencing the growth of *A. bovis*, P1. *A*, Stock Actinomyces medium. *B*, Basal medium plus coenzyme A plus uracil. *C*, Basal medium plus uracil. *D*, Basal medium. Basal medium was the same as stock medium with thiamine, guanine, xanthine, pyridoxal, pyridoxamine, and nicotinic acid being deleted. Inoculum was grown for 8 consecutive transfers on basal medium prior to this experiment. The inoculum which was a 48-hour culture was diluted to an optical density of 0.5, and one drop was used to inoculate each tube.

The various components of the medium which were expensive or difficult to obtain were then studied to determine which could be eliminated without reducing the growth promoting qualities of the medium. Of these only uracil was found to be required by *A. bovis* P1, and coenzyme A was stimulatory (Fig. 2). Of the added factors, pyridoxine, pyridoxal, xanthine, thymine, guanine, and nicotinic acid could be eliminated individually without affecting growth of either organism. But when these growth factors were eliminated simultaneously, growth of both organisms was invariably poorer either by

*As originally found by Puntoni[18] these cultures are sensitive to acid. Cultures should be placed at 5° C. before maximum growth is reached since the final pH of the medium may drop to 5.0 or 4.5.

colony count or growth rate. The individual effects of the remaining growth factors were not tested. The rationale for the remaining factors is as follows. Casitone has been reported to stimulate the growth of bovine strains of Actinomyces[5] and has been found to have a factor required for aerobic growth of *A. israelii* 296.[9] Cysteine is required for the growth of all strains of Leptotrichia and *A. israelii*.[6] On the basis of the results of Garrod[3] and Howell and Pine[5] starch is included in the medium. The yeast extract is included since it may fortify the medium with unknown growth factors. The pH 6.5 is recommended since there is little formation of phosphate precipitates at this pH. In preparation the basal medium may be filtered for sterilization, but equally good results are obtained with autoclaving. The medium may be stored in tubes under a pyrogallol-sodium carbonate seal for several months without deterioration of growth promoting activity.

Comparison of Stock Actinomyces Medium With General Laboratory Media Used for Growing Actinomyces.—Many experiments were designed to compare the growth promoting abilities of the casitone medium with Garrod's medium, with and without whole blood or serum, BHI agar in the presence or absence of whole blood, thioglycollate broth, or with modifications of some of these, the phosphate, cysteine, or glucose concentration being changed. It suffices to mention only the following results. BHI agar without blood supported the growth of more *A. bovis* and *A. israelii* colonies than the casitone medium. However, the colonies on BHI were limited in size and remained approximately 0.2 mm. in size, whereas the colonies on the casitone medium grew to 1 or 2 mm. in size. The casitone medium supported more rapid growth and was generally far superior to the other media.

Isolation of Actinomyces From Clinical Material.—In the isolation of Actinomyces from the 3 cases of human actinomycosis, several procedures which were based on the work of Rosebury and associates[11] were used. In all instances the sulfur granules were teased from the purulent material and freed from as much of the extraneous material as possible. The granules were then washed 4 or 5 times in sterile distilled water and then crushed with a glass rod in approximately 0.5 ml. of water. In the first case (*A. israelii* 895) the suspension was handled in the following manner. Brain heart infusion agar plates with and without 10 per cent whole blood were streaked and were then incubated in a Brewer jar filled with illuminating gas which was ignited. Thioglycollate tubes were also inoculated. In addition, stabs were made in Actinomyces stock medium having 0.7 per cent agar. These tubes were incubated with the usual pyrogallol-sodium carbonate seal. Within 48 hours pure cultures of *A. israelii* were found in these tubes. No growth was obtained within 5 days on BHI medium without or with blood or in the thioglycollate tubes. In the second case (*A. israelii* 534), streak plates were made on 4 media: BHI, BHI with 10 per cent whole rabbit's blood, Garrod's medium, and stock Actinomyces medium. The surface of several slants of stock Actinomyces medium were also streaked serially and then sealed with the pyrogallol-sodium carbonate seal. Within 18 hours the surfaces of the stock agar slants were

covered with minute adherent "spider" colonies. At 48 hours the plates were removed and a gross evaluation of the numbers of Actinomyces colonies was made by scanning the plates with the low-power objective of the microscope. No Actinomyces colonies were found on the BHI plates with or without blood, while the Garrod plates were given a rating of +3, compared with a +2 obtained with the stock Actinomyces medium.

In the case of *A. israelii* 699 initial isolations were made on stock medium only using both stabs and streaks on agar slants incubated with and without the pyrogallol-sodium carbonate seal. Growth occurred in 4 days in the anaerobic tubes only. Inoculum was taken from these tubes and suspended in sterile water to give a homogenous suspension, and one loopful was then used to streak BHI and stock Actinomyces plates.

Twelve single "spiderlike" colonies were then picked from the BHI plates and transferred to stock Actinomyces broth tubes. Similarly, 12 colonies were transferred to BHI broth. Of these, 12 colonies grew in the stock Actinomyces broth whereas only 2 grew in the BHI broth. In a second experiment 12 colonies were picked from BHI plates used for primary isolation. Of these, 6 were transferred to stock agar slants and 6 to BHI slants. Similarly, 12 colonies were picked from stock Actinomyces plates and 6 each were transferred to BHI and stock Actinomyces slants. In this second experiment, only those colonies transferred to stock Actinomyces slants grew even though the colonies may have been picked from BHI plates. The results obtained from human isolates clearly indicate that the stock Actinomyces medium is satisfactory as a primary isolation medium and far superior to BHI for secondary transfer and maintenance of *A. israelii*.

TABLE II. RESULTS OF ISOLATIONS OF ACTINOMYCES BOVIS ON SEVERAL MEDIA

CASE	MEDIUM*			
	BHI	GARROD'S	STOCK ACTINOMYCES	THIOGLYCOLLATE
	Primary isolation			
P2	+5, +3, –, +5	+3, +3, +1, –	+1, +1, +1, +1	Not tested
P3	+1, +3, +5, +5	+4, +3, +2, +1	+1, +1, +1, +1	Not tested
P4	1, 0, 0, 0	0, 0, 0, 1	5, 5, 10, 15	Not tested
	Repeated isolation after storage of pus in Deepfreeze for 14 days			
P2	+2, +2, +3, +5	5, +1, +1, +1	+5, +1, +1, +5	Not tested
P3	+1, +1, +2, +2	+1, –, –, +1	+1, +1, +1, –	Not tested
P4	33, 18, 40, 30	2, 0, 0, 4	8, 1, 6, 5	Not tested
	Repeated isolation after storage of pus in Deepfreeze for 30 days			
P2	+5, +3, –	+5, +5, +5	+3, +5, +3	+5, +5, +5
P3	+5, +5, +5	+5, +3, +5	+3, +5, +5	+5, +2, +5
P4	+5, +3, +4	+1, +1, –	+2, +1, +3	+1, +1, –

*Sulfur granules of each case were separated from the pus, washed 3 times in 0.05 per cent cysteine solution, ground, and homogenized with sterile glass rod; 1 loopful was streaked on each of 4 plates. With the exception of case P4 relative values based on the numbers of colonies observed were given to each plate. In general, a value of +5 indicates 50 to 100 colonies, a value of +1 indicates 2 to 11 colonies. In the first and second P4 isolations, the results of actual colony counts are given for each plate.

Table II presents the results of isolation from 3 cow heads having typical lumpy jaw. It is obvious from the results of Table II that, for primary isolation, BHI and Garród's medium are superior to the Actinomyces stock medium

insofar as numbers of colonies obtained. However, the isolations obtained with the stock Actinomyces medium were entirely satisfactory. The results of primary isolations obtained with serial dilutions tubes of the 3 media having 0.7 per cent agar was again in favor of BHI or Garrod's agar, more colonies were found at the greater dilutions (10^{-4}, 10^{-5}) of the inoculum in these media. However, repeated isolations of P1, P2, P3, and P4 from frozen pus made in these media and thioglycollate broth gave essentially the same results with all media.

It is also apparent from Table II that successful isolations may be made from the granules taken from purulent material which had been frozen for 30 days. Isolations of *A. bovis* (P1) have been made from granules frozen for a period of 6 months.

DISCUSSION

As a result of continuous work with many strains of Actinomyces under a variety of conditions and with many different media, it is concluded that the stock Actinomyces medium is a superior medium for the isolation, growth, and maintenance of these organisms. Of the media tested, it supports the maximum rate and amount of growth of the strains tested and maintains the viability of all strains. Frequent transfer of cultures is unnecessary and cultures of Actinomyces may be stored at 5° C. for 1 to 6 months without loss of viability. Although it permits completely satisfactory isolation from clinical material it is not necessarily the best medium for primary isolation. Our results, like those of Garrod,[3] indicate that no one medium of those tested is unquestionably superior. The results indicate that on primary isolation or when the inoculum originates from heavy growth of Actinomyces, brain heart infusion agar permits rapid growth of more single cells into minute colonies. Yet, when these single microscopic colonies are transferred to BHI medium, these colonies do not grow. Clearly, some nutrilite present in the clinical material or large inoculum is depleted on the secondary transfer. On the other hand, on primary isolation or when the inoculum originates from heavy growth, the stock Actinomyces medium does not support the widely dispersed growth of individual colonies obtained with the BHI agar. Yet, repeated experiments with both human and cattle strains have shown that single colonies picked from either BHI or stock Actinomyces agar plates invariably grow to a discernible size within 6 to 10 days when transferred to stock Actinomyces medium, broth, or agar. Therefore, the value of the casitone medium rests primarily on its ability to maintain growth of Actinomyces after primary isolation. Alternation between several media to maintain cultures of Actinomyces as described by Rosebury and co-workers[11] and Hazen and Little[4] is not necessary when the stock Actinomyces medium is used. The fact that in agar stabs and semisolid medium it supports much more rapid and reliable growth of Actinomyces has led to our use of the medium exclusively for isolation and subculture from clinical and experimental material. In such tubes growth is invariably observed within two days.

It is recommended that where possible the composition of the medium should be retained as given in the formula. Coenzyme A may be eliminated without seriously affecting the growth of the test Actinomyces strains. For routine use, the medium with 1.5 per cent agar is tubed in slants and in agar deeps sufficient for pouring plates. For stab cultures or dilution tubes 0.7 per cent agar is used. The tubes of medium may be sealed with the pyrogallol-sodium carbonate seal and stored at room temperature until needed. Tubes stored for 3 to 4 months have apparently supported as good growth as that obtained with fresh media.

SUMMARY

A laboratory medium satisfactory for the isolation and maintenance of Actinomyces is described. This medium has been compared to brain heart infusion agar and Garrod's starch medium for maintenance and isolation of Actinomyces from clinical material. The Actinomyces medium is superior for maintenance and allows maximum growth of all the strains tested. It also supports excellent growth of *Corynebacteria acnes, C. liquefaciens, Lactobacillus bifidus,* and *Leptotrichia buccalis.*

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FAR ULTRAVIOLET DIRECT PHOTOMETRY APPLIED TO PROTEIN ELECTROPHORESIS

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DIRECT photometry is the generally preferred method of protein quantitation for routine zonal methods of electrophoresis. The staining procedures commonly employed, however, have certain limitations for quantitative purposes. These include the influence upon the staining of a number of variable factors which must be controlled, such as drying, denaturation, salt concentration, and pH.[1] The staining may also depend upon the individual protein, and questions have been raised concerning the ability to rinse the stain from the background without losing any from the protein.[2]

An electrophoretic method already described employs as a medium, a thin layer or film of buffer, stabilized by such a low concentration of gelling agent that it does not gel but remains as a clear fluid.[3] This or other electrophoretic procedures whose backgrounds are sufficiently transparent to light may thus permit the proteins to be followed during the course of their migration and quantitated by means of direct ultraviolet absorption, without staining. Adaptation of ultraviolet photometry to filter paper electrophoresis of proteins has been previously reported by Kimbel[4] and by Bracco, Magistretti, and Rubino.[5] However, their results appeared limited, by the absorption and reticular structure of the paper. This background absorption is largely eliminated by the use of a clear, fluid buffer solution. A simple method of following proteins qualitatively during preparative electrophoresis has already been presented.[6] The extension of the method to protein quantitation is described below.

MATERIALS AND METHODS

The electrophoresis was set up and run as previously described,[3] except for the following modifications: (1) a tris buffer (consisting of 0.05 moles of tris(hydroxymethyl) aminomethane and 0.05 moles of LiCl per liter, adjusted to pH 8.6 with HCl) was substituted for the veronal buffer, due to the ultraviolet absorption of the latter, and (2) a quartz plate, instead of plastic, was used to layer the buffer over for the same reason. Two 6-inch quartz squares* were cemented together to form a 6 by 12 inch cell bottom, and glass walls were cemented along the edges to form a container so the buffer solution would not run out.

The photometry was conducted by exposing a photographic plate, directly under the quartz cell, to light coming down from above, through the protein solution in the cell. The latter was raised about ⅛ of an inch above the platform it was on by means of glass strips placed under the ends adjacent to the electrode vessels. This permitted photographic plates to be slid underneath from the side, without disturbing the electrophoresis. The light was

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*Thermal American Fused Quartz Company, Dover, N. J.

obtained from a Bausch & Lomb grating monochrometer (Catalog No. 33-86-40-01) at a wave length setting of 200 mμ. The exit slit width was set at 3 mm. and the entrance slit width at 5 mm. The monochrometer was positioned 6 feet above the quartz cell.

The plates were developed in Kodak D-19 developer for 4 minutes at 20° C., rinsed in 1 per cent acetic acid for 30 seconds (except the Kodak SWR plates which were rinsed in running water for 2 minutes), and fixed in Kodak fixer F-5 for 5 minutes at 20° C.

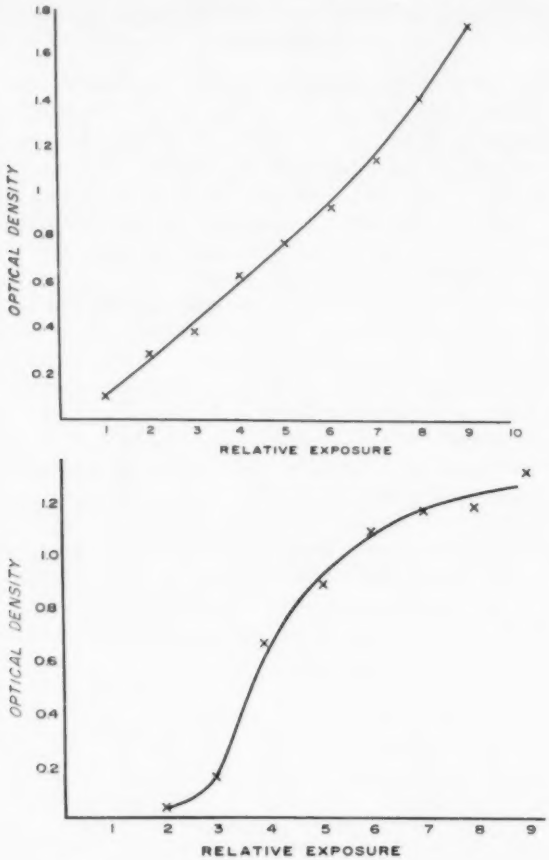

Fig. 1.—Relation of optical density on processed photographic plate to relative exposure (in units of entrance slit width openings, millimeters). Top is for Kodak spectrum analysis No. 1 U. V. plates, 1-minute exposures, and bottom is for Kodak SWR plates, 8-second exposures.

RESULTS

Calibration curves were obtained by measuring in a Kelab densitometer* the optical density of a series of graded light exposures. The exposures were varied by changing the setting of the entrance slit width (before the light reached the monochrometer). The exit slit width was held constant at 3 mm.

*Kelab AB, Stockholm, Sweden.

The results are shown in Fig. 1 for 1-minute exposures with Kodak spectrum analysis No. 1 U. V. plates, and for 8-second exposures with Kodak SWR plates. In each instance, all the values were obtained on a single photographic plate.

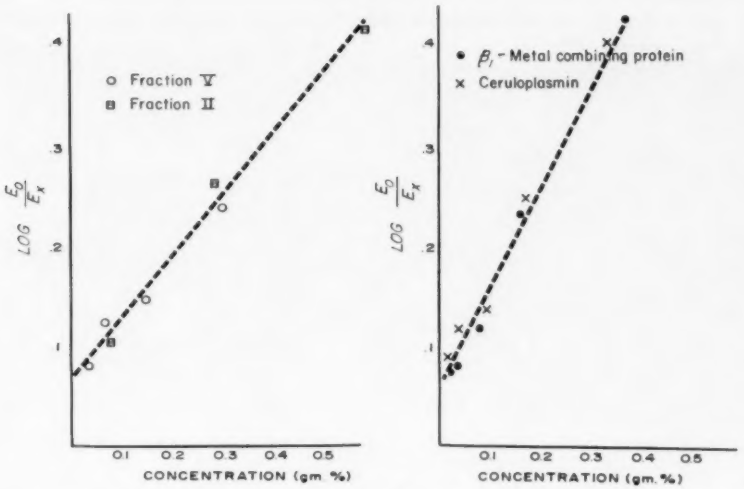

Fig. 2.—Relation of calculated value of log $\frac{E_0}{E_x}$ to concentration for various serum proteins.

The applicability of Beer's law was then investigated by exposing for 1 minute a spectrum analysis No. 1 U. V. plate under a quartz cell. The cell had been partitioned into sections each of which held a different serum protein solution, 1.7 mm. thick and with tris buffer containing 160 mg. per cent agar used as a solvent. The optical density on the processed photographic plate corresponding to each solution was then measured in a densitometer and converted graphically (Fig. 1) into relative exposure values. Since the relative exposure (E) is a measure of the intensity of the light (I) transmitted through the protein solution, the $\log \frac{E_x}{E_o}$ can thus be substituted for the $\log \frac{I_o}{I_x}$ in Beer's law (E_o and I_o referring to the solvent and E_x and I_x referring to the protein

TABLE I. REPRODUCIBILITY OF MULTIPLE DETERMINATIONS ON A SINGLE SAMPLE
PER CENT OF TOTAL PROTEIN

	ALBUMIN AND α_1-GLOBULIN	α_2-GLOBULIN	β-GLOBULIN	γ-GLOBULIN
	52.88	13.71	12.00	21.41
	53.41	13.43	12.36	20.80
	49.83	15.08	12.97	22.11
	48.79	16.52	13.40	21.29
	50.32	15.34	12.32	22.01
	47.83	14.96	13.83	23.37
	46.83	16.79	14.45	21.93
	47.40	15.19	12.97	24.48
Mean	49.66	15.13	13.04	22.18
Standard deviation	2.29	1.23	0.77	1.13

solution). The results are shown for solutions of human fractions V* and II* on one plate and for solutions of human β_1-metal-combining protein† and ceruloplasmin,† on a second plate (Fig. 2). The intercept on the ordinate represents absorption due to the tris buffer and agar gelling agent. The relation of optical density to concentration for the same 4 proteins was also determined after suitable dilution by means of a Beckman DU spectrophotometer at a wave length of 213 mμ, and the results are shown in Fig. 3.

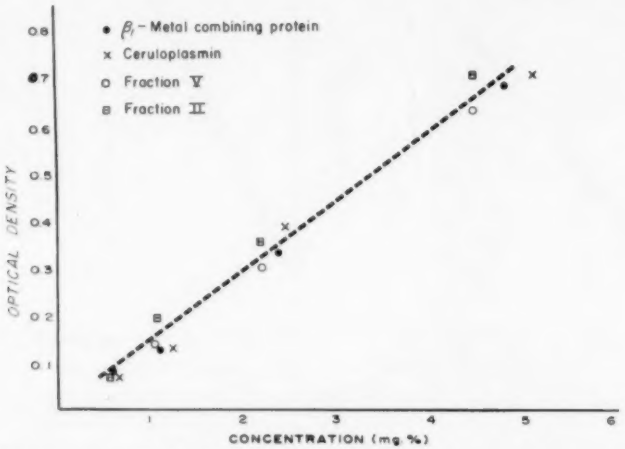

Fig. 3.—Spectrophotometric measurement of the absorbancy versus concentration for the same proteins used in Fig. 2.

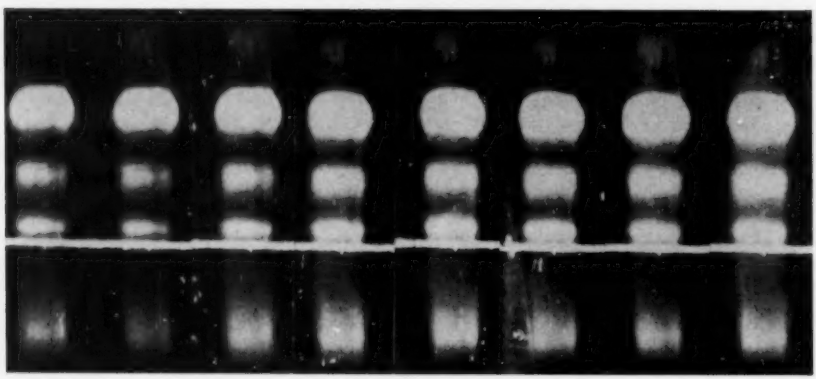

Fig. 4.—Eight processed photographic plates obtained from the same serum electrophoretic separation.

Fig. 4 presents 8 photographic exposures of a single serum electrophoretic separation. The per cent of each protein was determined by calculating the

*Obtained from the American Red Cross.
†Obtained from the Michigan State Department of Health.

$\log \frac{E_o}{E_x}$ values along the migration path, as above, and measuring the per cent of the total area of the curve under each peak (Table I).

The ability to follow the proteins during the migration without disturbing the electrophoresis is illustrated in Fig. 5.

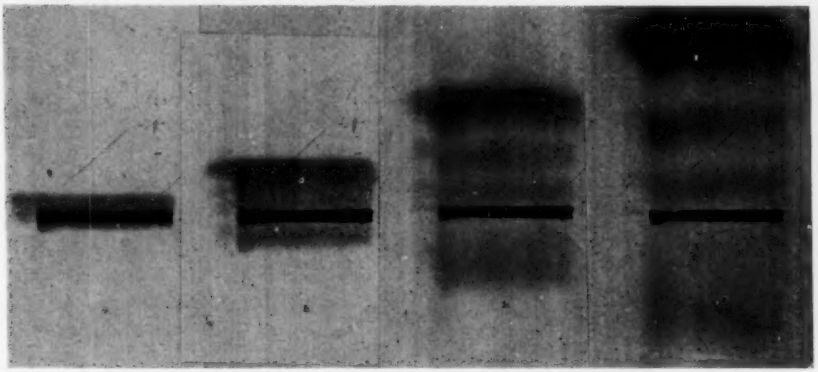

Fig. 5.—Printed plates of a single serum separation after running times, left to right, of 17, 45, 90, and 150 minutes.

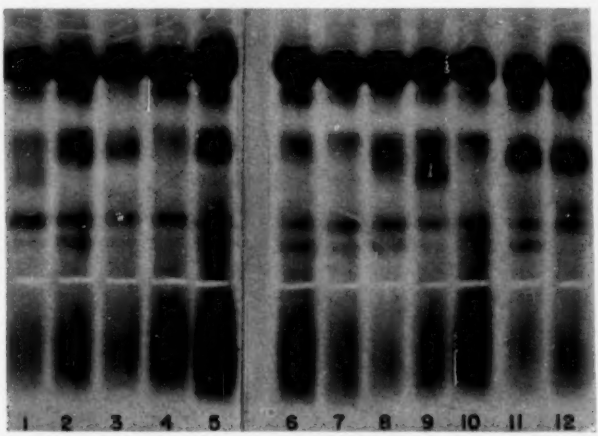

Fig. 6.—Electropherograms of 12 different sera, after drying and staining.

Fig. 6 shows the electropherograms of 12 different sera, run simultaneously for routine clinical purposes, after drying and staining. They were run for a period of 5 hours as previously reported,[3] except that the staining was done with the more sensitive Amido black 10B, rather than bromphenol blue.[7] Different samples of the same sera were also subjected to electrophoresis in the tris buffer for 3½ hours, after which the photometry was conducted by the ultraviolet method on Kodak SWR plates (Fig. 7). The curves relating the

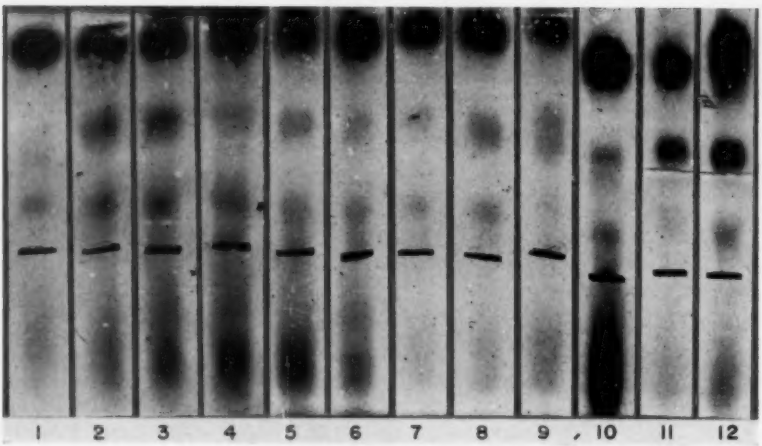

Fig. 7.—Electropherograms of the same sera as in Fig. 6, after ultraviolet photometry and printing.

Fig. 8.—Absorbancy of stain along migration path for the electropherograms of Fig. 6, shown on the left. The calculated values of log $\frac{E_o}{E_s}$ along the migration path for the same sera in corresponding position, obtained from the plates of Fig. 7, are shown on the right.

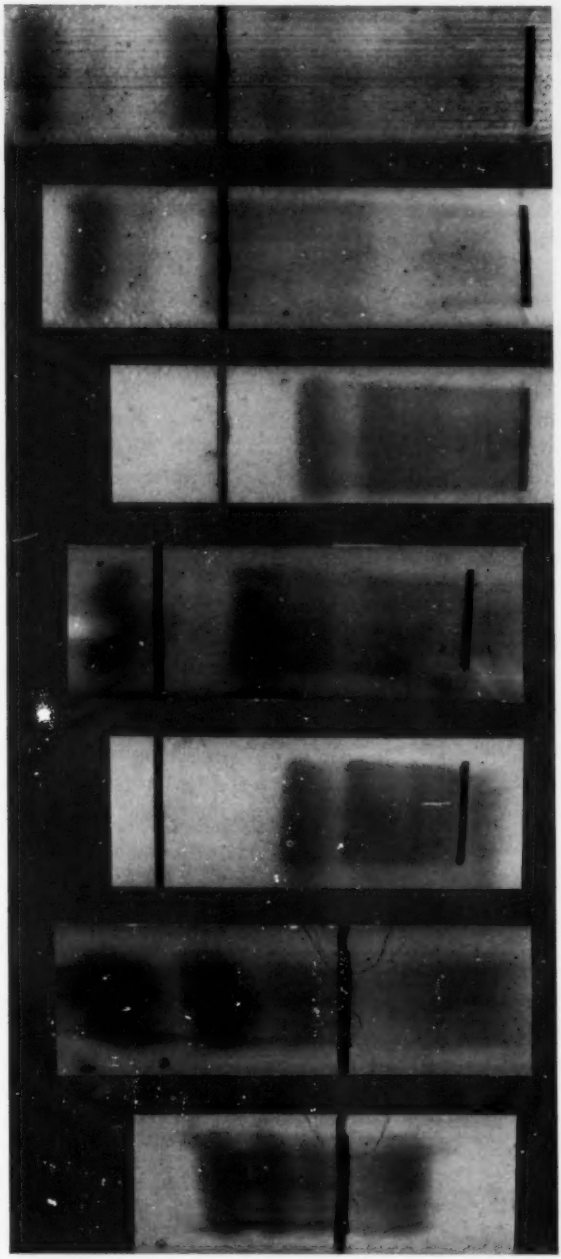

Fig. 9.—Fluid film electrophoresis on the same serum, with gelling agents and running times as follows (left to right): 0.16 Gm. per cent agar at 1½ and 3 hours 1.0 per cent Cato 8 (a starch derivative of the National Starch Products, Inc.) at 1½ and 3 hours, and commercial potato starch at 1½, 3, and 3½ hours.

absorbancy of Amido black 10B stain along the migration path are compared to the calculated values of $\log \frac{E_o}{E_x}$ obtained from ultraviolet absorption, in Fig. 8.

Although agar has been used in most of the preliminary work, the fluid method appears to be general, and a number of other gelling agents can be used to stabilize the buffer as a clear fluid, a few of which are compared in Fig. 9. All were dissolved by heating in the buffer to a boil, and the photometry was conducted on Kodak spectrum analysis No. 1 U. V. plates.

DISCUSSION

The results presented in Fig. 2 show that the $\log \frac{E_o}{E_x}$ is linear with protein concentration, in conformity with Beer's law. The values for fractions V and II, on a single photographic plate, indicate that they have similar absorption coefficients, as do β_1-metal-combining globulin and ceruloplasmin, on a second plate. When different exposures are compared on the same photographic plate, variations due to drifts in light source intensity or differences between emulsions or processing tend to cancel out. Thus the different proteins in the same serum sample, compared to each other on the same plate (as done in Table I from Fig. 4), act as an internal standard for each other. This method of internal control is analogous to methods commonly employed in spectrographic analysis,[8] and the reproducibility (Table I) appears comparable to that obtained with other electrophoretic procedures.[9]

The absorbancy values obtained by means of the Beckman DU spectrophotometer (Fig. 3) for fractions V and II, β_1-metal-combining globulin, and ceruloplasmin show that the absorption coefficients are similar for all 4 proteins in this wave length region. The results are consistent with the findings of Goldfarb and associates,[10] that a large part of the absorption around 200 mμ is due to the peptide linkage, thus accounting for the similarity in absorption coefficients.

When setting up the photometry, blank photographic plates should be exposed to light from the monochrometer, to ascertain whether the uniformity of the background density is sufficient for the purposes at hand. Variations due to the light source can be reduced by decreasing the slit width openings or increasing the distance between the monochrometer and photographic plate. It is preferable to run the proteins in the direction of constant rather than changing wave length.

The emulsions of most photographic plates investigated had a good degree of uniformity. The least uniform were Kodak SWR plates, which are the only ones that do not require a coating with a fluorescent material at 200 mμ. Any emulsion can be coated, either so ordered or done with Eastman ultraviolet sensitizing solution. In the case of SWR emulsions, it may be necessary to read E_o values along a path without protein, adjacent and parallel to the migration, although films may be more uniform than the plates investigated. The necessity for darkening the room during the photometry can be eliminated by the use of

slow emulsions, usable in room light, if desired. Exposure times can readily be determined which will give readable density values for the various proteins, although it is a simple matter to take 2 exposures of different duration when a sufficiently broad concentration range may be encountered. When set up, the method is rapid and simple to perform, and a large number of samples may be run and exposed simultaneously on a single photographic plate or film.

Prealbumins are often observed with the fluid film method, and some of the sera in Fig. 6 show 2 or 3 prealbumins. The resolution is better than that of the corresponding ultraviolet electropherograms in Fig. 7. This may be due in part to the greater running time and distance of migration of the stained patterns. The distance of migration was not sufficient to clearly separate the α_1-globulin from albumin (Fig. 4) which were arbitrarily measured together.

The resolution also depends upon the chemical composition, pH and ionic strength of the buffer, the sensitivity of the photometric procedure, and the nature of the gelling agent. Although proteins have much greater absorption coefficients at 200 mμ than at 280 mμ, the use of light at a wave length of 185 mμ, and containing less stray radiation, would result in a considerable increase in sensitivity.

The gelling agent employed affects not only the resolution attained, but also the relative mobilities,[11] as can be seen from a comparison of the electropherograms of Fig. 9. The use of different gelling agents and buffer combinations for ultraviolet photometry is being investigated.

SUMMARY

A method is described of photographic photometry in the far ultraviolet field for following and quantitating serum proteins during electrophoresis. The various protein fractions investigated appear to have similar absorption coefficients at the wave length used, and conformation to Beer's law was obtained. Comparing the different proteins in a serum sample on the same photographic plate tends to cancel out a number of variables, and the reproducibility is comparable to other electrophoretic procedures.

Twelve different sera were subjected, after electrophoresis, to both staining and far ultraviolet photometry, and corresponding results were obtained. Factors affecting the resolution of the method are discussed.

The ultraviolet photometry is rapid and simple to perform, and a large number of samples can be run and exposed on a single photographic plate or film. The variable factors involved in staining procedures are eliminated since the photometry is direct, and no drying or staining is involved.

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A SIMPLIFIED METHOD FOR THE DETERMINATION OF CITRIC ACID

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MANY methods have been described for the determination of citric acid in biologic fluids. Procedures consisting of the conversion of citric acid to pentabromacetone and its colorimetric determination appear to be used most widely. As originally described,[1] this method consisted of the following steps: (1) removal of "interfering substances" by heating with sulfuric acid and the addition of bromine water, (2) oxidation of citric acid to pentabromacetone by permanganate in the presence of bromine, (3) extraction of pentabromacetone into petroleum ether, (4) development of color by reaction of the pentabromacetone with sodium sulfide, and (5) measurement of the intensity of the color developed.

The original procedure has been modified by many investigators. Iodide,[2-4] thiourea-borax,[5, 6] thiourea-borax-sulfide,[7] and alkaline pyridine[8, 9] have all been used as color-developing reagents. Vanadic acid has been used as an oxidizing agent in the place of permanganate.[7, 9-11] In the methods employing pyridine to develop color, the removal of interfering substances by heating with sulfuric acid has been omitted; only β-hydroxybutyric acid, acetoacetic acid, and trichloroacetic acid and other halogenated hydrocarbons give false positive readings.

We have investigated several of the methods which have been described. Because of its apparent simplicity, we attempted to carry out citrate determinations according to the method of Perlman, Lardy and Johnson[12] that is described in a widely used laboratory manual.[13] The results were very erratic and the color developed with sodium sulfide was found to be extremely unstable. The precautions given by Perlman and associates[12] were investigated. These studies revealed that (1) neither pyridine nor dioxane, claimed to stabilize the color produced with sodium sulfide, had any stabilizing effect on the color; the same observation was made in the case of pyridine by Natelson, Lugovoy, and Pincus[14]; (2) an excess of H_2O_2 or permanganate did not influence the results within a wide range; (3) acid washing of the petroleum ether had no effect; and (4) the pentabromacetone was quite stable in the petroleum ether layer. Erratic results were obtained not only when several aliquots of a citric acid solution were analyzed, but also when color was developed from a single preparation of pentabromacetone in petroleum ether. It was apparent, therefore, that the difficulty occurred in the color development stage. Natelson and associates,[14]

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who also encountered difficulty with this method, demonstrated that color stability was dependent on temperature. Even when the temperature was controlled, however, the results were obtained, although somewhat better, were still unsatisfactory.

Natelson and co-workers[5] have developed a sensitive, precise micromethod for color development using a thiourea-borax reagent. This method requires boiling the samples in an oil bath, special glassware, and special spectrophotometer modification to give a long light path. We have been able to achieve considerable simplification in the determination of citric acid in biologic fluids by eliminating tedious treatment with sulfuric acid or other agents to remove interfering substances, a step which we found to be unnecessary in most instances, and, by adapting procedures described by others to standard laboratory glassware, eliminating the necessity for extra long spectrophotometer cuvettes and special vessels with ground glass stoppers. This has been accomplished without sacrifice of precision of specificity, and the number of determinations which can be carried out by a single technician has been increased several fold.

MATERIALS AND METHODS

Reagents.—

Sulfuric acid: Approximately 50 per cent sulfuric acid (v/v) is prepared by adding 1 volume of concentrated sulfuric acid, reagent grade, to 1 volume of water.

Potassium permanganate solution: Water is saturated with potassium permanganate at room temperature.

Weak permanganate solution: Saturated permanganate solution diluted 1:15.

Potassium bromide: A 1 M solution is made in distilled water.

Petroleum ether: Hexane (Skelly, solve B) or n-heptane has proved satisfactory, as obtained from the manufacturer. We routinely wash petroleum ether in acid by shaking it with several aliquots of concentrated sulfuric acid. The remaining acid *must* be removed by washing with water several times. Drying of the ether is not necessary. Equally good results have been obtained using the petroleum ether as obtained from the manufacturer, but the acid-washing technique has been used routinely because of possible variations between lots.

All of the above reagents are stable for long periods of time at room temperature.

Thiourea-borax: Two grams of sodium borate, reagent grade, and 4 Gm. of thiourea are dissolved in water and brought to a volume of 100 ml. This is stable for several days at 4° C.

Three per cent H_2O_2: Prepared from 30 per cent H_2O_2 (Superoxyl) and stored at 4° C.

Citrate standard: Citric acid is dissolved in 1 N H_2SO_4 to give a solution of 1.000 mg. per milliliter. This stock standard is stable indefinitely at 4° C. A 1:10 dilution is made, and citric acid determinations are carried out on 0.5, 1.0, and 2.0 ml. aliquots to give 50, 100, and 200 γ standards, respectively.

Procedure.—A sample containing between 10 and 2,000 μg of citric acid is placed in a tube marked at the 10 ml. level. Blood sugar tubes have been found most satisfactory. Five-tenths milliliter of 50 per cent sulfuric acid is added and the volume is adjusted to approximately 5 ml. with distilled water. Two-tenths milliliter of potassium bromide and 1 ml. of potassium permanganate solution are added. The mixture is shaken briefly and allowed to stand at room temperature for from 5 to 20 minutes.

The tubes are chilled in an ice bath, and 3 per cent H_2O_2 is added dropwise until the permanganate is decolorized. Approximately 20 drops are usually required. Toward the end of this titration, at least 30 seconds should be allowed to elapse between the addition of drops of H_2O_2, otherwise overtitration will result. Sufficient "weak permanganate solution" is added until a faint violet or yellow-brown color persists for more than a few seconds. The total

volume is adjusted to exactly 10.0 ml. with water. Exactly 13.0 ml. of petroleum ether is added, the tubes are stoppered tightly with rubber stoppers, and are shaken for 5 minutes. Exactly 10.0 ml. of the petroleum ether layer is added to 10.0 ml. of the thiourea-borax solution in a Coleman Junior 25 mm. cuvette. The cuvette is stoppered tightly with a rubber stopper and shaken for 5 minutes. It is then centrifuged slowly for a few minutes to break any emulsion that may have formed. Photocolorimetric readings are made against a reagent blank in a Coleman Junior spectrophotometer at 445 and 650 mμ. The optical density at 650 mμ is subtracted from that at 445 mμ to correct for turbidity and/or cuvette error. Duplicate standards containing 50, 100, and 200 μg of citric acid are analyzed with each determination. If the color produced by the sample exceeds that of the 200 μg standard, an aliquot of the remaining pentabromacetone in the petroleum ether layer must be diluted in petroleum ether and the color development step repeated to bring the reading within the range of the calibration curve. The color is stable at room temperature for several hours. Even after standing overnight at room temperature, only 5 to 10 per cent fading of color occurs.

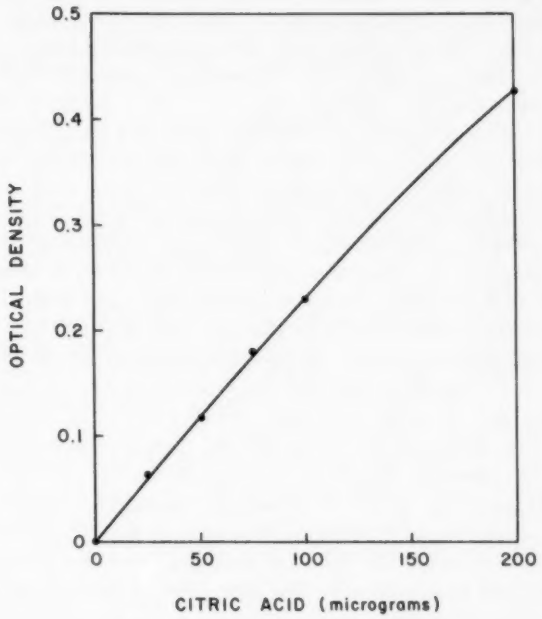

Fig. 1.—Typical citrate standard curve.

RESULTS

A. Calibration Curve.—The calibration curve follows Beer's law in the 0 to 50 or 75 γ range, but subsequently the increase of the optical density values is usually somewhat less than predicted (Fig. 1). This appears to coincide with the development of a small precipitate at the interface between the aqueous and petroleum ether layer when amounts greater than approximately 50 μg are analyzed. Nonetheless, accurate results may be obtained up to approximately 200 μg, and by dilution of the petroleum ether layer, the range may be extended to at least 2,000 μg.

B. Recoveries.—Excellent recoveries of added citrate are achieved from rat and human urines, human blood plasma (a trichloroacetic acid extract is used), and homogenates of rat liver and kidney.

C. Specificity and Interfering Substances.—Studies have been carried out to determine whether, in high concentration, a variety of substances gives false readings as citrate and to determine whether these substances interfere with the determination of small quantities of citric acid.

The compounds studied were isocitric acid, *cis*-aconitic acid, glucose, acetone, acetic acid, lactic acid, α-ketoglutaric acid, β-ketoglutaric acid, heparin, and acetaldehyde. Citrate determinations were carried out on 5,000 μg aliquots of these compounds with and without the addition of 50 μg of citric acid. Parallel determinations were carried out on samples that had been treated to remove interfering substances, as has been suggested by Perlman and associates. This treatment consists of diluting the total volume to approximately 5 ml. with water, adding 0.5 ml. 50 per cent H_2SO_4, and placing in a boiling water bath for 10 minutes. After cooling, 0.5 ml. of saturated bromine water is added; the citrate determination is then carried out.

All of the compounds tested, with the exception of β-ketoglutaric acid and heparin, gave negative readings for citric acid with or without preliminary treatment to remove interfering substances. β-Ketoglutaric acid gave positive readings for citric acid, the values obtained ranging from 50 to 100 per cent of the value obtained with equal weight of citric acid. No reading was obtained when β-ketoglutaric acid was treated with hot sulfuric acid and bromine water, and it was found that treatment with hot sulfuric acid alone sufficed. Heparin gave a reading of about 0.4 per cent of that obtained with an equal weight of citric acid. Treatment to remove interfering substances had no effect on the reading obtained. The readings obtained are so small that no measurable effect on plasma citrate level results from the use of heparin in obtaining blood samples, even if it is used in considerable excess. None of the substances tested interfered with the recovery of citric acid except for acetaldehyde. In the presence of 5,000 μg of acetaldehyde, incomplete recovery of citric acid was observed. A 50 μg sample gave readings of approximately 40 μg. However, if the sample was treated with hot sulfuric acid and bromine water, complete recovery of citric acid was achieved. The same effect was achieved by merely placing the sample with sulfuric acid in a boiling water bath for 5 minutes.

Toluene or formalin may be used as a preservative for urine samples without interfering with the citric acid determinations. Urine and plasma samples have also been analyzed for citric acid, both with and without preliminary treatment with heat and sulfuric acid and bromine water (Table I). No significant difference has been observed, even when urine from a subject in severe diabetic ketosis was studied in this way.

D. Reproducibility.—Replicate determinations have been carried out on standards, urine, and plasma samples. The standard deviations obtained in these replicate determinations are presented in Table I.

TABLE I. RESULTS OF REPLICATE CITRATE DETERMINATIONS

SAMPLE	NUMBER OF REPLICATES	MEAN CITRATE CONTENT (μG)	STANDARD DEVIATION (μG)
50 μg standard	8	50.00*	5.94
50 μg standard	8	50.00*	1.91
50 μg standard	8	50.00*	1.06
100 μg standard	8	100.00*	1.88
100 μg standard	8	100.00*	2.52
100 μg standard	8	100.00*	3.84
200 μg standard	8	200.00*	4.82
200 μg standard	8	200.00*	6.50
200 μg standard	8	200.00*	5.07
Urine A (from a normal subject after ingestion of citrate)	8 (0.05 ml. aliquots)	1,510 /ml.	67
Urine B (from a normal subject after injestion of citrate)	8 (0.10 ml. aliquots)	1,855 /ml.	61
Urine C (from a normal subject after injestion of citrate)	8 (0.02 ml. aliquots)	1,606 /ml.	57
Urine D (from normal subject)	8 (0.10 ml. aliquots)	809.7 /ml.	10.6
Urine E (from normal subject)	8 (0.10 ml. aliquots)	1,781 /ml.	20
Urine E, pretreated with hot H_2SO_4 and bromine water	8 (0.10 ml. aliquots)	1,769 /ml.	34
Urine F (from normal subject)	8 (0.20 ml. aliquots)	132.6 /ml.	4.1
Urine F, pretreated with hot H_2SO_4 and bromine water	8 (0.20 ml. aliquots)	129.5 /ml.	4.8
Urine G (from a diabetic patient in severe ketosis)	8 (0.10 ml. aliquots)	1,078 /ml.	29
Urine G, pretreated with hot H_2SO_4 and bromine water	8 (0.10 ml. aliquots)	1,104 /ml.	62
Plasma A (from a normal subject)	6†	23.51/ml.	0.67
Plasma B (from a patient with hemochromatosis)	8†	25.84/ml.	0.56
Plasma B, pretreated with hot H_2SO_4 and bromine water	8†	24.97/ml.	0.37
Plasma C (from another patient with hemochromatosis)	8†	13.88/ml.	0.38
Plasma C, pretreated with hot H_2SO_4 and bromine water	8†	14.09/ml.	0.32

*By definition.

†Five milliliters of a trichloroacetic acid extract prepared by adding 3 ml. plasma to 4 ml. 20 per cent trichloroacetic acid.

DISCUSSION

A simple and accurate method for the determination of citrate has been developed by combining a rapid method for the conversion of citric acid to

pentabromacetone with a color developing reagent that produces a stable color at room temperature with a high degree of specificity. It has thus been possible to eliminate time-consuming treatment of samples to eliminate interfering substances. It is apparent that the necessity for such treatment depends upon what agent is used to develop color. Sodium sulfide seems to lack, to a considerable degree, specificity in reacting with pentabromacetone. This is true even when treatment with hot sulfuric acid and bromine water is carried out. Iodide seems to have little advantage over sodium sulfide except for the development of more intense color. Pyridine, while apparently much more satisfactory as a color developing reagent than either sodium sulfide or iodide, requires that the oxidation of the citric acid be carefully controlled and the development of color be carried out under conditions of fixed temperature and time.[5] Thiourea-borax, on the other hand, is sufficiently specific so that even if the tedious treatment to remove interfering substances is omitted, very few compounds interfere with the determination. Virtually identical results are obtained with urine, tissue, and plasma samples, whether or not treatment is carried out with hot sulfuric acid and bromine water. If the presence of large amounts of β-ketoacids or acetaldehyde is suspected, the interfering effect may be removed merely by brief heating in a boiling water bath.

Further simplification is achieved by the use of rubber stoppers, notwithstanding the admonition[6] that these react with thiourea. Worn stoppers may result in the appearance of a yellow color in the petroleum ether phase, but this does not interfere with the spectrophotometer readings, which are carried out on the aqueous layer. Thus, it has been possible to use standard glassware and to develop color directly in cuvettes. Only 2 tubes are used in the entire procedure; pentabromacetone is formed and extracted into petroleum ether in the first tube and the color is developed and measured in the second. As a result, a single technician, with the aid of a mechanical shaker, can perform accurately 30 to 40 citrate analyses in the course of a working day, an increase of several fold over older methods.

SUMMARY

A modification of earlier methods for the determination of citric acid in biologic fluids is described. Citric acid is converted to pentabromacetone, and color development is carried out with a thiourea-borax reagent. The entire procedure may be carried out simply and rapidly using standard laboratory glassware.

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A CORRECTION FOR THE INTERFERENCE OF UREA IN THE DETERMINATION OF TOTAL ALPHA AMINO ACID NITROGEN IN PLASMA BY THE NINHYDRIN PHOTOMETRIC METHOD

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COLORIMETRIC methods for the determination of total plasma amino acids have not been completely satisfactory.[1-3] They were at greatest variance in the presence of increased concentrations of urea. In 1947, Chinard and Van Slyke[4] showed that the manometric carbon dioxide method was the only one which gave reliable results. The ninhydrin method as modified by Moore and Stein[5, 6] gave accurate and reproducible results in measuring quantities of specific amino acids. An attempt was made to adopt this procedure to determine total plasma alpha amino acids. Certain theoretical objections were apparent: ammonia is an interfering substance but at normal values contributes less than 1 per cent of the total amino nitrogen. In liver disease with hepatic coma, ammonia might contribute as much as 6 to 8 per cent of the total amino nitrogen. A correction can be made for this by measuring the blood ammonia levels. Creatinine does not add significant color even at pathologic levels and uric acid, which interferes with other colorimetric methods, does not react with ninhydrin. Urea adds significantly to the measured value of total alpha amino nitrogen, even if moderately elevated, and therefore a correction is necessary to compensate for its interference.

TABLE I. RECOVERY OF ALPHA AMINO NITROGEN ADDED TO PLASMA AS DETERMINED BY PHOTOMETRIC NINHYDRIN METHOD

SAMPLE	UNCORRECTED PLASMA (MG. PER 100 ML.)	PLASMA PLUS AMINO ACID (MG. PER 100 ML.)	AMINO ACID ADDED (MG. PER 100 ML.)	AMINO ACID RECOVERED (MG. PER 100 ML.)	RECOVERY (PER CENT)
Plasma B_1 + glutamic acid	6.58	8.97	2.50	2.39	96
Plasma B_2 + glutamic acid	6.58	11.53	5.00	4.95	99
Plasma 15 + glutamic acid	3.86	8.68	5.00	4.82	96
Plasma H* + glutamine	5.95	10.81	5.00	4.86	97
Plasma 22 + glutamine	4.69	7.04	2.50	2.35	94

*Glutamine standard used after 3 weeks at 4° C.

MATERIALS AND METHODS

Measured amounts of leucine, glutamic acid, and glutamine were recovered completely from aqueous solutions or when added to plasma, the average recoveries being 98 per cent (see Table I).

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The color contribution of urea was studied as follows. Aqueous urea solutions ranging in concentration from 9.5 to 95 mg. per 100 ml. urea nitrogen were prepared. In addition, various amounts of urea nitrogen were added to normal and uremic plasmas (47* and 94* mg. per 100 ml. urea nitrogen added), as well as to plasma filtrates (19, 38, and 95 mg. per 100 ml. urea nitrogen added). When these solutions were analyzed, it was found that the color produced followed Beer's law. (See Fig. 1.) The color yield compared to leucine was 0.03. The slopes in Fig. 1 showed that each 10 mg. per 100 ml. urea nitrogen increased the value of total amino acid nitrogen by 0.14 mg. per 100 ml.

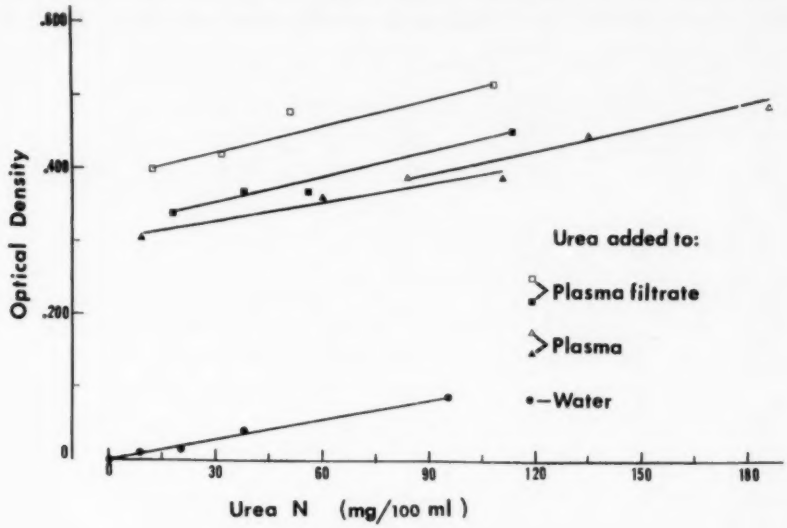

Fig. 1.—Urea correction curve.

It was found that both plasmas stored in a CO_2 icebox and plasma filtrates refrigerated at 4° C. were stable for at least 4 weeks.

Procedure.—To 2 ml. of plasma in a 50 ml. conical centrifuge tube is added 10 ml. of 1 per cent picric acid. The tubes are shaken, centrifuged, and a 10 ml. aliquot of the supernatant fluid† is transferred to the resin columns of Dowex 2-X8,‡ as outlined by Moore and Stein.[5-7] The effluates are collected in a 100 ml. volumetric flask and diluted to the mark with distilled water. Duplicate 1 ml. aliquots are pipetted into matched 18 by 150 mm. Pyrex culture tubes. One milliliter of ninhydrin reagent is added with a Cornwall syringe.[6] The tubes are then covered with aluminum caps, shaken for about 10 seconds, and placed in a rapidly boiling water bath for exactly 15 minutes. They are then cooled rapidly to room temperature. Five milliliters of 50 per cent ethanol is added with a Cornwall syringe. The tubes are fitted with rubber stoppers and shaken by machine for about 1 minute. The optical densities are then read within one hour on a Coleman Junior spectrophotometer at 570 mμ. A standard curve was prepared using 1 ml. aliquots of leucine solutions ranging in concentration from 0.04 to 0.2 mM. per liter, and a table was prepared for converting the optical density directly into concentration.[5, 6] For a blank, duplicate 2 ml. of water instead of plasma is carried through the entire procedure.

*These values were corrected for plasma water in plotting the slopes in Fig. 1.

†An alternate method is to pour the entire supernatant fluid onto the column, rinsing the tube and precipitate with 0.02 N HCl.

‡The resin is prepared as outlined on page 916 of reference 7, except a 3 to 4 cm. resin bed was used to ensure complete removal of all picric acid.

A blood urea nitrogen must be determined for each plasma analyzed.[11] Before use, all equipment must be thoroughly rinsed first with tap water and then with distilled water to remove traces of ammonium salts and oxidizing substances.

RESULTS AND DISCUSSION

The mean of 23 normal values for total alpha amino nitrogen of plasma was 3.7 ± 0.5 S.D. mg. per 100 ml., as compared to a mean of 20 published normal values (by the manometric CO_2 method) of 4.1 ± 8 S.D. mg. per 100 ml.[8, 10] (See Table II.) Ten plasmas (4 normal, 6 abnormal) were analyzed

TABLE II. ALPHA AMINO NITROGEN OF PLASMA (MG. PER 100 ML.)

SAMPLES	BUN	PHOTOMETRIC NINHYDRIN		MANOMETER NINHYDRIN		B/D %	DIAGNOSIS
		UNCORRECTED (A)	CORRECTED (B)	UNCORRECTED (C)	CORRECTED* (D)		
Normal							
1	13.1	4.76	4.57				
2	11.2	3.97	3.81				
3	16.0	4.66	4.44				
4	11.1	3.82	3.65				
5	16.9	4.31	4.07				
6	8.2	4.20	4.10				
7	13.4	3.63	3.44				
8	14.0	3.75	3.54				
9	12.0	3.05	2.88				
10	14.1	4.48	4.26	4.58	4.48	95	
11	19.0	4.08	3.83	4.12	4.02	95	
12	9.8	3.12	2.99	3.47	3.37	89	
13	18.4	3.89	3.64	3.98	3.88	94	
14	9.6	2.30	2.16				
15	17.4	3.86	3.61				
16	18.0	3.90	3.64				
17	17.0	3.87	3.65				
18	12.0	3.68	3.50				
19	16.2	3.96	3.76				
20	11.3	3.72	3.55				
21	20.6	4.12	3.82				
22	21.2	4.69	4.41				
23	12.0	3.45	3.26				
Mean		3.9	3.7		3.9	95	
S.D.		±0.6	±0.5		±0.5		
Mean				Published values	4.1	90	
S.D.					±0.8		
Abnormal							
A	50.0	4.34	3.64				Diabetic with Kimmelsteil-Wilson syndrome
B_1	85.0	5.18	4.07				Malignant hypertension
B_2	107.0	6.23	4.65				Malignant hypertension
C	46.0	4.81	4.23				Essential hypertension
D_1	84.0	4.27	3.09				Essential hypertension
D_2	92.0	4.37	3.06				Essential hypertension
D_3	73.5	3.91	2.87		3.10	93	Essential hypertension
E	24.2	4.64	4.30				Essential hypertension
F	53.5	4.80	4.05		4.24	96	Chronic pyelonephritis
G	65.5	4.76	3.82		4.10	93	Nephrosclerosis
H	133.0	5.95	4.00		4.38	91	Polycystic renal disease
I	35.1	2.79	2.31		2.58	90	Gangrene of extremity
J	19.3	3.75	3.51		3.56	99	Juvenile diabetic possibly early Kimmelsteil-Wilson syndrome

*Assume 0.1 mg. amino nitrogen due to BUN for normal values only.

by both methods. All the colorimetric values were in satisfactory agreement with the manometric values, showing the validity of the urea correction as well as the reliability of the method for the measurement of total plasma alpha amino acids in normal subjects and in those with elevated blood urea nitrogens.

It will be noted from Table II that all values, when compared with the manometric method, were lower by 2 to 11 per cent (average 6 per cent). This can be explained by two facts:

1. The amino acid proline, comprising about 7 per cent of the circulating amino acids, is not measured by the colorimetric method because it lacks an alpha amino nitrogen.

2. All amino acids do not have a 100 per cent color yield when compared to leucine. It was calculated from published data that the aggregate of amino acids yields about 96 to 97 per cent color.[7]

SUMMARY

A colorimetric method is described for the determination of total alpha amino acid nitrogen in plasma. A standard ninhydrin procedure was used with a correction for the presence of urea. Results obtained are in satisfactory agreement with the manometric carbon dioxide method. The relative simplicity and rapidity of the procedure make it suitable for large numbers of determinations.

We should like to thank Dr. William Stein for his invaluable advice and review of the manuscript and Mr. George Ross for review of the manuscript.

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Addendum.—Since submitting this manuscript, 1 ml. of plasma and 5 ml. of picric acid have been used, pouring entire contents onto the column as outlined and collecting in 50 ml. volume flasks. The results have been the same.

A NEW INSTRUMENT FOR THE MEASUREMENT OF ELECTRICAL CONDUCTIVITY OF BODY FLUIDS

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A REVIEW of the medical literature of this country reveals no study of the electrical conductivities of the body fluids. The lack of a suitable instrument for making these studies may have contributed to the absence of investigation in this field.

Because of the theoretical ease in making electrical conductivity measurements on minute samples, and the freedom of error in reading the results, it was decided to construct a simple Wheatstone bridge electrical conductivity measuring apparatus for minute samples. After the author's suggestion, Worden and White* constructed a Wheatstone bridge for the measurement of electrical conductivities in relative milliequivalents per liter of sodium chloride of samples of electrolytes in volumes less than 0.166 ml.

Description of Instrument.—The block diagram of this instrument is shown in Fig. 1. The schematic diagram is shown in Fig. 2.

The completed Wheatstone bridge, amplifier, rectifier, meter, sampling pipette, and balancing control are mounted in a suitable case as shown in Fig. 3.

Two arm electrodes are placed on top of the case to receive in a horizontal position the plastic pipette used to collect the fluid sample for electrical conductivity testing. These arm electrodes are spaced so that they will be in contact with the ring electrodes on the sampling pipette as shown in Fig 3. Two platinum electrodes extend from the ring electrodes of the sampling pipette into the lumen of the pipette. A meter and a balancing control arm are positioned on top of the case. The balancing control has been calibrated in electrical conductivities to those determined for aqueous solutions of sodium chloride of known milliequivalents per liter concentrations. The instrument is therefore direct reading for samples in relative electrical conductivities of milliequivalents per liter of sodium chloride. The sampling pipette is made of plastic tubing 10 cm. in length, and the electrodes are 5 cm. apart. The volume of fluid necessary for the test is approximately 0.166 ml.

An alternating bridge current is used in order to avoid the complicating effects of electrode polarization. A simple amplifier, rectifier, and meter are used to indicate the bridge balance point. For the practical balancing point, the balancing arm control is adjusted until a minimum reading is shown by the

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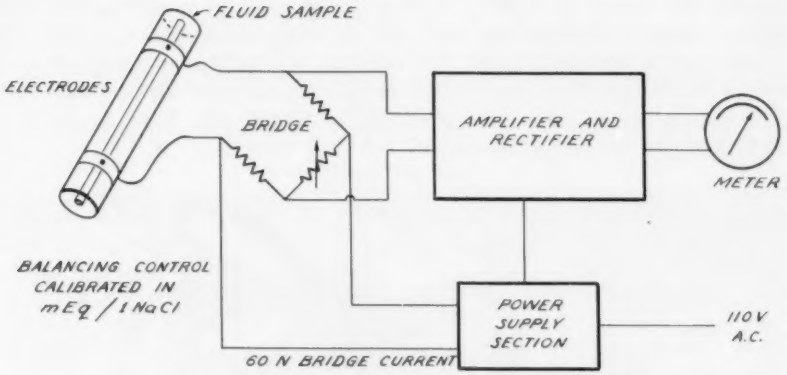
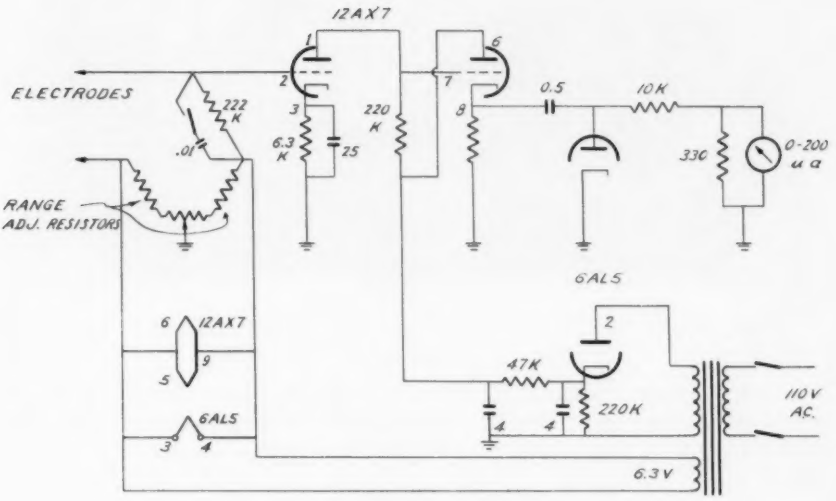
*Sam Worden and Gayle White of the Texas Industrial Instruments Company and the Humble Research Laboratories, respectively.



BLOCK DIAGRAM OF CONDUCTOMETER

Fig. 1



SCHEMATIC DIAGRAM

Fig. 2

meter. This indicates that the bridge is balanced and that the reading of the balancing arm control is correct. A number of readings were made on commercially prepared Darrow's solution and Hartmann's solution as a check on the consistency of the instrument. All readings for the Darrow's solution were identical, and the readings for the Hartmann's solution were identical also.

In the use of the conductivity measuring bridge, care must be taken to exclude air from the column of sample.

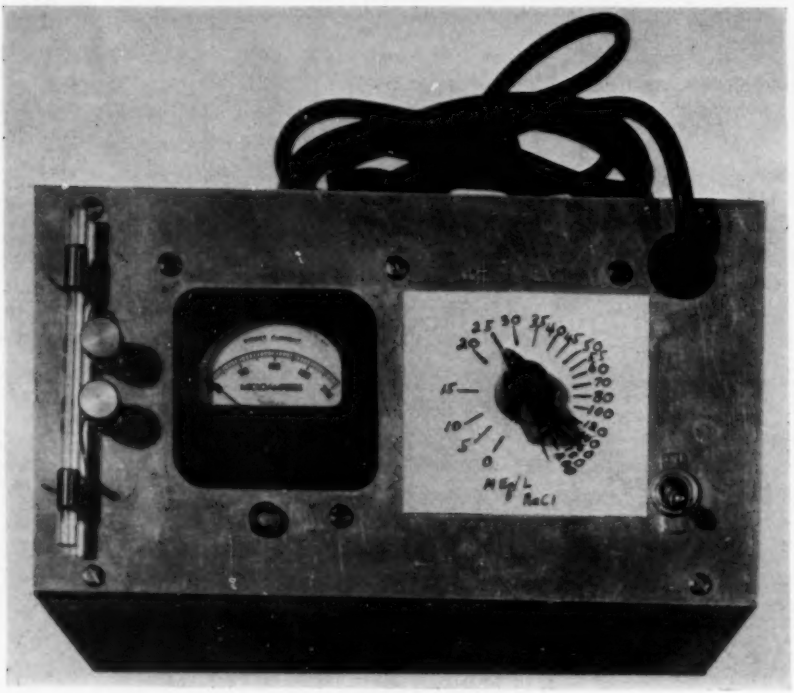


Fig. 3.

SUMMARY

A description is given of a new instrument constructed for the rapid and simple determinations of electrical conductivities of small amounts (less than 0.166 ml.) of body fluids which is relatively free of methodical error.

A NEW SCREENING TEST FOR FIBROCYSTIC DISEASE OF THE PANCREAS

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IN ANOTHER manuscript a new electrical conductivity measuring Wheatstone's bridge was described.[1] Using this instrument it was found that the electrical conductivity of the saliva of the normal individual appeared to be reduced after stimulation whereas that of the patient with fibrocystic disease of the pancreas did not. The purpose of this study is to show the variation in these findings.

TABLE I. ELECTROLYTE CONDUCTIVITIES OF SALIVA

PATIENT	SEX	AGE IN MONTHS	ELECTRICAL CONDUCTIVITY IN EQUIVALENT MEQ./L. NaCl	
			BEFORE STIMULATION	AFTER STIMULATION
Patients With Fibrocystic Disease of the Pancreas				
Goss	F	108	25	40
Yates	M	48	18	50
Yates	M	48	44	45
Smith	F	84	25	35
Franklyn	F	36	40	44
Hill	F	48	38	40
Groves	M	24	30	40
Ferguson	M	14	60	70
		Mean	35	46
Normal Subjects				
Speights	F	28	45	28
Duty	F	51	50	28
Pincoffs	F	89	25	25
Dorlac	M	108	30	25
Coombs	F	36	28	30
Harrison	M	77	30	20
Barrier	F	53	30	22
Gray	F	26	27	25
Gambino	F	36	40	33
Rosenthal	M	48	36	34
Koster	F	96	34	28
Foitik	M	14	25	22
		Mean	33	27

METHODS

Collection of saliva samples was made during periods of nonstimulation and after stimulation. Stimulation was provided by the nursing of a dry nipple or, in older patients, by the chewing of a stick of Dentyne gum. A stick of Dentyne gum cut in small pieces and placed in a small amount of distilled water shows no electrical conductivity. Examinations were made on initial samples, and samples obtained after 6 minutes of stimulation. The results of these studies and the comparison with the normal are shown in Table I.

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RESULTS

The children with fibrocystic disease of the pancreas for the most part were patients at one time or another in The Texas Children's Hospital. All of these children had clinical features of the disease and laboratory confirmation by sweat tests, stool trypsin, fat, starch determinations, duodenal intubation for trypsin and viscosity, and chest x-ray studies. The normal children were studied in routine examinations in private office practice.

It is recognized that the electrical conductivity values of the saliva in this study represent the sum provided by all the electrolytes in that solution. In addition to sodium chloride, saliva contains potassium chloride, sodium bicarbonate, sodium phosphate, calcium carbonate, calcium phosphate, potassium iodide, and potassium sulphocyanate.[2] The resulting electrical conductivities of saliva found in this study and expressed in relative values of milliequivalents per liter of pure sodium chloride will be for the most part due to the sodium chloride which is the chief electrolyte present. Buffering may reduce the total values, hence, changes in electrical conductivities under stimulation will represent changes in the concentration of sodium chloride and/or its ionization.

From Table I it is seen that the electrical conductivity of the saliva of the normal individual is remarkably uniform, and after stimulation tends to decrease. Contrary to this are the results shown also in Table I of the functioning of the salivary glands of the patient with fibrocystic disease of the pancreas. Saliva secreted under stimulation tends to remain high in electrical conductivity values if initially high, and to become high if low initially. This would indicate that the control mechanism governing the secretion of electrolytes in the saliva of the patient with fibrocystic disease of the pancreas is defective under stimulation, but may be adequate when no stimulation is present in some of those patients. Whether this test may be used also to evaluate the severity of the disease remains to be determined in further studies.

The high or increased electrical conductivity found in the saliva, when stimulated, of patients with fibrocystic disease of the pancreas is thought at present to be of value in the easy and simple screening of this disease, being adaptable both to office and hospital practice.

SUMMARY

The electrical conductivity of the electrolytes in the saliva of the patient with fibrocystic disease of the pancreas tends to remain high or increase with stimulation of the salivary glands in contrast to the normal which tends to decrease. The sampling and timing of the sampling of the saliva of patients appears to be a practical office and hospital method for the screening of the patient with fibrocystic disease of the pancreas.

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EVALUATION OF DIFFERENT SUBSTRATES FOR THE STAPHYLOCOCCAL COAGULASE TEST AND A COMPARISON OF THE TUBE AND THE SLIDE TECHNIQUES

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AT THE present time at least 4 substrates are readily available to the routine diagnostic laboratory for the purpose of performing coagulase tests on staphylococcal isolates. Two of these substrates are in a dehydrated form and are available commercially as Diagnostic Plasma (Warner-Chilcott Laboratories), a dried human plasma, and Bacto-Coagulase plasma (Difco Laboratories), a desiccated rabbit plasma. The remaining two available substrates are fresh human plasma and fresh rabbit plasma. Since the coagulase test is of considerable importance as a criterion of pathogenicity of staphylococci, it is of practical interest to know if these 4 substrates give comparable results when performing the test.

Cruickshank[1] found that coagulation occurred in human, rabbit, horse, ox, sheep, and guinea pig plasma with his strains of staphylococci but a definitive, comparative study as to preference was not done. Chapman[2] has stated that human plasma takes longer to clot than does rabbit plasma. Also, clots of human plasma are less firm than are those of rabbit plasma. Again, a definitive study was lacking. Recently, Waller[3] and Boyd[4] have conducted comparative studies using fresh human plasma and dehydrated human plasma. The former found that the dehydrated product gave a higher percentage of positive findings than did the fresh plasma. Boyd, however, stated that the two substrates gave identical results.

In view of the lack of definitive, comparative information concerning the performance of these 4 substrates in the coagulase test, we decided to test a large number of staphylococci freshly isolated from human infections in each plasma. Also, since the method of testing for coagulase is of importance both the tube test and a modified slide technique were used.

MATERIALS AND METHODS

The staphylococci used in this work were all isolated from infectious processes of hospitalized human beings. Abscesses were the most common type of infection involved. The cultures were isolated and identified by the usual laboratory methods and were tested for

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coagulase activity soon after isolation. Most of these cultures produced a golden pigment on isolation but some did not. As Fairbrother[5] and others have pointed out pigment production is not a reliable criterion for pathogenicity.

The dried plasmas used in these tests were reconstituted according to the directions of the manufacturer and were used within an hour or two, maximum, after rehydration. Fresh human plasma was obtained from blood bank material (not pooled) and fresh rabbit plasma was obtained by cardiac puncture of rabbits (not pooled). The latter two plasmas were either used soon after collection or were stored at −20° C. after collection and thawed just before use.

For performing the tube test, 0.5 ml. of an overnight broth culture of the organism was inoculated into 0.5 ml. of fresh (undiluted) or rehydrated plasma. The contents of each tube were mixed and the tubes incubated at 37° C. Each tube was observed after 2, 4, and 6 hours and again after overnight incubation. Any degree of clotting, whether minute or massive, was considered a positive test.

The slide technique used in this study was a modification of an older technique in which one suspicious colony was emulsified directly in plasma, or emulsified in saline or water to which plasma is added. If mixtures of coagulase-positive and coagulase-negative colonies were present in the material being examined, a false negative result might be obtained if the wrong colony was chosen for the test. In our modification a sterile cotton swab is rubbed lightly but firmly across the surface of a blood agar plate containing colonies of staphylococci. An emulsion is made by immersing the swab in a small tube containing 0.5 ml. of sterile saline, rotating the swab in the saline a few times to produce a heavy homogenate. Thus, we have found that even in the presence of a few coagulase-positive colonies in a predominantly coagulase-negative population a strong positive test will result. Another advantage to this method is that a series of suspensions may be made by the technician and set aside until a large number of tests are to be performed. When the test is conducted a Petri dish or a glass plate is marked off into areas with a wax pencil, one drop of each respective suspension is placed within one area, and one drop of plasma is added. The plate is rotated a few times and each area observed macroscopically for clumping. With positive cultures immediate clumping occurs, whereas spontaneous or rough clumping will take place before the drop of plasma is added.

TABLE. I. COMPARISON OF SUBSTRATES FOR COAGULASE TESTING

METHOD	FRESH RABBIT PLASMA	DEHYDRATED RABBIT PLASMA	FRESH HUMAN PLASMA	DEHYDRATED HUMAN PLASMA
Tube				
number pos.	92	92	40	55
per cent pos.	100	100	44	60
Slide				
number pos.	92	92	92	92
per cent pos.	100	100	100	100

RESULTS

In a preliminary investigation 50 strains of staphylococci freshly isolated from human infections were compared for coagulase activity in fresh rabbit plasma and in dehydrated human plasma (Diagnostic Plasma). We found, as Chapman had stated, that the rabbit plasma clotted much more firmly and more readily than did the human plasma. Of greater interest was the fact that only 1 of these 50 strains was negative with the rabbit plasma, whereas 15 of the 50 strains failed to produce any coagulum in the human plasma.

This stimulated the more wide-scale study with more strains of staphylococci using 4 substrates and the two techniques mentioned. In this phase of

the work a total of 139 freshly isolated staphylococci were tested. Of these, 47 were coagulase negative in all substrates by both the tube and slide methods. Comparative findings with the remaining 92 cultures are shown in Table I.

It is obvious that both the substrate and the method influence the results. Whereas all 92 cultures gave a positive test in the slide technique with all substrates, only the rabbit plasma gave comparable results with the tube method. Moreover, when both the rabbit and human plasmas were positive in the tube test the rabbit plasma invariably clotted faster and more firmly.

An additional 44 staphylococcal isolates were tested using only the slide technique with all 4 substrates. All 44 were positive regardless of the substrate.

DISCUSSION

The findings in the present study emphasize that both substrate and method are important in coagulase testing. With the commonly used tube technique rabbit plasma appears to be more reliable than human plasma, both in regard to rapidity and in ease of reading results and in percentage of positive findings. We have found no direct proof that only human plasma will give information regarding pathogenicity of staphylococci for human beings. All of the cultures used in this work came from human infections, thus we assume that they played a role in the pathogenesis of these diseases. It is not reasonable to assume that those cultures that were coagulase negative in human plasma but coagulase positive in rabbit plasma are nonvirulent for the human subject. Even though rabbit plasma presumably contains more of the specific clotting factors necessary to the coagulase reaction we do not feel that this leads to false positive results.

In addition to the animal species from which plasma is obtained, other factors relating to the test will affect the result obtained. Thus, plasmas from certain hosts within species are deficient in clotting factors and will not yield positive results even with known coagulase-positive organisms. This emphasizes the need for including a positive control with each batch of plasma to be used. Often an old plasma is not effective nor is one containing an excess of anticoagulant. We have found that freezer storage of fresh plasma at –20° C. apparently does not impair the clotting mechanism. The time interval at which results are read is another factor affecting the outcome of the test. Clots may be lysed after too long an incubation period, emphasizing the need for early and frequent readings. When using the tube technique minute degrees of coagulation may be overlooked, a factor largely eliminated by the use of the slide method. It is the general consensus that any clotting, however small, is to be considered as a positive test. Whether or not those strains producing early and massive clotting are more virulent than those producing minimal coagulation is not known. Still another factor in the coagulase test is the selection of colonies for the test. When mixtures of both coagulase-positive and coagulase-negative colonies are present on a plate it is possible that a negative colony will be picked

leading to a false negative result. This factor is eliminated by using several colonies for the test instead of a single colony, whether the tube or the slide technique is used.

We feel that the slide method outlined in this paper is preferable to the usual tube technique for several reasons. First, it is rapidly done and lends itself to mass screening in the busy laboratory. Second, it is more economical since small amounts of plasma will suffice. Third, results are available very quickly. Fourth, minute coagulation is more readily seen that with the tube test. Fifth, our findings indicate that human plasma gives more reliable results when the slide method is used. The reason for the last difference in the two methods is not clear.

As a final point it should be emphasized that dried rabbit plasma was as effective as was fresh rabbit plasma and dried human plasma was as effective as was fresh human plasma. Since these two dehydrated products are readily available and presumably of uniform consistency their use is indicated for the coagulase test if the slide technique is used. If tubes are used rabbit plasma is preferable, whether dried or fresh.

SUMMARY

In comparing large numbers of staphylococci freshly isolated from human infections for coagulase activity it was found that rabbit plasma was superior to human plasma when using the conventional tube technique.

When a revised slide technique was used both rabbit and human plasmas gave equivalent results.

Dehydrated plasmas reacted about the same as did fresh plasmas under all circumstances.

The advantages of the slide technique over the tube technique are discussed.

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THE CELLULOSE METHOD FOR COLLECTING SWEAT

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THE discovery by di Sant'Agnese in 1953,[1-3] that children with cystic fibrosis of the pancreas have an abnormally high concentration of chloride and sodium in their sweat provided us with an important diagnostic and research tool. Several methods for collecting and analyzing sweat for its electrolyte content have been described.[3-6] The method reported here is simpler and more accurate.

The sweat test commonly employed today consists of collecting trunkal sweat on a 2 by 2 in. cotton gauze sponge of known weight by sealing it against the skin under a small piece of waterproof sheeting. Then the patient is subjected to a mild thermal stress, usually by placing him in a plastic bag. The gauze is recovered and reweighed establishing the weight of the sweat sample. The sweat is eluted from the gauze with distilled water and an aliquot is examined for chloride and sodium concentration. This method is time consuming and introduces the errors of sample dilution. Another technique[6] collects total body sweat on plastic sheeting. However, factors such as emotional sweating and accumulated skin debris which can be less well controlled make total body sweat a less reliable fluid for analysis than sweat from a small standard site.

MATERIALS AND METHODS

Unless otherwise stated the experience reported is based on 200 sweat tests on 194 patients. The tests were conducted between April 15, 1958, and Sept. 12, 1958, on patients seen at the Ohio Tuberculosis Hospital, Ohio State University Hospital, Ohio State University Medical Out-Patient Clinics, normal volunteers, and selected relatives of patients with cystic fibrosis. Patients were selected because of their medical diagnosis. Patients with varying degrees of respiratory and cardiac insufficiency were included. The age ranged from 9 months to 87 years with a mean of 41.3 years. Eighty-six per cent were white and 56 per cent were men. Almost all patients had a similar "partial-pay" socioeconomic status. Ninety-three and one half per cent of the tests were performed in the same room which was heated by electric heaters. All glassware was washed, triple rinsed in redistilled water, and oven-dried.

The test is performed by dressing the patient in two piece pajamas and placing him on a hospital bed in a preheated room. The back and abdomen are washed to the point of faint erythema with 4 by 4 in. cotton gauze pads which are saturated with distilled water. The skin is fanned dry. To both sides of the abdomen and back a piece of cellulose sponge*

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*The cellulose sponge is the common household cellulose sponge. The dry sponge is firm and can be easily cut on a slicer. New sponges were cut to size, rinsed 10 times in distilled water, and dried in an oven at 120° F. for 3 hours.

1 by 1¾ by 3/32 in. is attached to the prepared skin by covering it with a 3½ by 3½ in. piece of Saran Wrap plastic sheeting, the edges of which are sealed to the skin by waterproof tape.

The patient is then placed in a waterproof bag* which is large enough to accommodate the body, except for the head, and allows some freedom of motion. The bag is drawn snugly about the neck by a drawstring and one or two woolen blankets are placed over the patient.

A period of 60 to 70 minutes in the bag is usually adequate to produce a sample. Following this the bag is removed and the cellulose sponges are recovered. The sponge containing the sweat is then placed in a 10 ml. glass syringe and the plunger is inserted compressing the sponge. If the syringe is capped, the sample may be briefly stored without contamination or evaporation. The sweat is easily recovered by compressing the sponge to a volume of 0.1 to 0.2 ml. The sweat is collected in a small test tube and electrolyte determinations are made.

Occasionally the sample volume from a given site is too small, and samples from two or more sites are combined. Dilution of a sweat sample is occasionally required to provide an adequate total volume of 0.6 to 0.7 ml. Dilutions greater than 1:2 are not required.

Chloride is measured by the method of Schales and Schales[7] which requires 0.1 to 0.2 ml. of sweat. Sodium is determined by flame photometry using 0.5 ml. of sweat which is diluted with 0.02 per cent Sterox solution to a 1:50 or 1:100 dilution depending on the value of the chloride determination. A flame photometer standard curve must be constructed from stock solutions of known sodium concentration over a range from 10 to 180 mEq. per liter. The appropriate portions of this curve should be verified with standard solutions each time a test is performed.

RESULTS

In 95 per cent of the first 100 serial tests the sweat sample volume was sufficient to allow an analysis of chloride and in 87 per cent the sodium was also computed. In only 5 per cent of the tests was the sample volume too small to permit testing for either chloride or sodium. Sample volume data are summarized in Table I.

TABLE I

SAMPLE VOLUME DATA USING BILATERAL TEST SITES, INITIAL 100 SERIAL TESTS	PER CENT OF CASES	
	ADEQUATE	INSUFFICIENT
1. Sample volume for Cl	95	5
2. Sample volume for Na	87	13
3. From abdomen for both Cl and Na	65	11
4. From abdomen for Cl alone	89	11
5. From back for both Cl and Na	82	10
6. From back for Cl alone	90	10

The electrolyte concentration in each of the 4 samples obtained from a given patient compared closely for both chloride and sodium. The reported value was the average of the concentration obtained from the different sites. The sodium concentration was usually greater than the chloride; the mean showed that sodium was greater than chloride by 4.5 mEq. per liter in the initial 100 tests.

The age of the patient did not significantly influence the volume of the samples produced, since adequate samples were consistently produced from

*Sweat bags were made of Tomac Nylonite sheeting, available from the American Hospital Supply Company. This sheeting is waterproof, soft, autoclaveable, will drip-dry, and serves as a vapor barrier.

both age extremes. The age of the patient was not a factor in the electrolyte concentration, nor did any age range tend to be higher or lower in concentration. The application of dry heat did not increase the sample volume produced. It was noted that the rate of sweating did not correlate with the electrolyte concentration in the sweat. Likewise the volume of the sample obtained did not influence the electrolyte concentration.

The oral temperature was measured before and at the conclusion of the diaphoretic period. An average change in oral temperature of + 0.72° C. occurred with a range of – 0.2 to + 2.5° C.

In order to determine the accuracy, reproducibility and the effect of certain physical factors on the test, several studies were performed.

To determine the per cent error of the test and the possible effect of the sponge on the concentration of the electrolytes, 1.0 ml. of a stock solution of sodium chloride of known concentration was added to several sponges and determinations of the electrolyte concentration of the recovered solutions were made. This was repeated for several different saline concentrations. The results are summarized in Table II.

TABLE II

CONTROL SALINE ADDED TO THE SPONGE		CONCENTRATION OF RECOVERED SOLUTIONS			
		CHLORIDE		SODIUM	
mEq./L.	NO. OF TESTS	AVERAGE (mEq./L.)	PER CENT ERROR	AVERAGE (mEq./L.)	PER CENT ERROR
15	5	15.5	+ 3.3	14.6	– 2.7
25	6	25.5	+ 2.0	24.6	– 1.6
55	5	55.7	+ 1.3	56.2	+ 2.2
85	6	81.6	– 4.0	81.4	– 4.2
110	6	107.8	– 2.0	111.4	+ 1.4
125	6	125.7	+ 0.6	121.2	– 3.0
150	6	149.0	– 0.7	146.9	– 2.1
180	6	180.8	+ 0.4	176.9	– 1.7
	Total 46	Average	+ 0.1%	Average	– 1.5%

In order to determine the reproducibility of the sweat electrolyte concentration in a given patient, 15 patients had more than one sweat test as described in Table III.

The effect of room temperature on the diaphoretic time was noted in the first 200 serial tests. The relationship is described in Table IV.

DISCUSSION

In the past the major disadvantage of the commonly employed di Sant'-Agnese sweat test has been the time consuming manipulation of the cotton gauze pads on which the sweat was collected and the elution of the sweat from the cotton. Methods yielding pure sweat samples have been collections of total body sweat or sweat collected over a large body area which introduces the additional variable factors of skin cleanliness, accumulated skin debris, emotional factors of sweating and skin secretions from sources other than sweat. The method described obviates all weighing and elution and provides for the local collection

TABLE III

PATIENT	TEST NO.	DATE	CHLORIDE mEq./LITER	SODIUM mEq./LITER
A. H.	12	4/22/58	5.2	15.8
	118	8/12/58	15.9	22.8
E. H.	26	5/ 6/58	92.9	117.1
	27	5/13/58	79.1	102.2
B. B.	39	6/30/58	90.8	92.6
	227	9/19/58	55.9	56.1
C. M.	90	7/26/58	39.7	37.3
	252	9/30/58	66.0	79.8
W. H.	98	7/30/58	75.5	73.1
	106	8/ 6/58	71.1	71.1
J. S.	99	8/ 1/58	38.3	47.2
	152	8/23/58	35.9	44.2
C. I.	141	8/19/58	85.6	98.2
	217	9/17/58	83.9	105.9
C. M.	144	8/20/58	76.6	76.7
	233	9/18/58	64.8	68.8
G. G.	146	8/21/58	62.3	74.9
	160	8/26/58	no sample	no sample
	175	9/ 3/58	38.2	44.7
	188	9/ 9/58	accident	39.3
C. B.	154	8/23/58	100.4	125.9
	225	9/19/58	102.8	114.1
	293	10/28/58	105.1	126.0
R. S.	163	8/28/58	103.4	121.8
	285	10/25/58	94.7	119.7
E. R.	257	10/ 5/58	12.7	14.3
	267	10/12/58	12.9	19.7
	273	10/19/58	8.5	—
	287	10/26/58	12.0	20.5
S. Z.	258	10/ 5/58	22.5	24.3
	266	10/12/58	24.5	25.3
	274	10/19/58	14.4	13.6
	286	10/26/58	25.2	28.3
M. B.	259	10/ 5/58	20.6	23.7
	265	10/12/58	45.1	54.0
	272	10/19/58	21.0	29.0
	289	10/26/58	24.4	30.9
K. L.	260	10/ 5/58	41.6	48.7
	264	10/12/58	37.7	45.1
	288	10/26/58	42.3	52.7

TABLE IV

THE RELATIONSHIP BETWEEN ROOM TEMPERATURE AND THE DIAPHORESIS TIME, 200 SERIAL TESTS

ROOM TEMPERATURE	AVERAGE TEST TIME (MIN.)
70-75° F.	172
76-79° F.	124
80-84° F.	102
85-89° F.	86
> 89° F.	67

of pure sweat from any accessible body site. The cellulose sponge method and the cotton gauze method have been run simultaneously. The cellulose sponge method reduces the technical time of the test by 50 per cent.

A sample volume large enough for analysis of chloride was obtained in 95 per cent of the tests when four sample sites were used. The values obtained from the different trunkal sites compared closely. The test was uniformly well tolerated with no untoward reactions noted. Patients with respiratory or cardiac insufficiency tolerated the test without complication. However, in 5 per cent of tests an insufficient sweat sample was obtained, and this was due to one of the following causes: insufficient test time, active diuresis in progress, dehydration, or a cool room. If the room was not warm or diuresis was in progress, extending the test time did not produce sweating. The room temperature was found to be the single most critical factor in the volume of the sweat samples produced. Large samples were produced at room temperatures of 89 through 91° F. If the room temperature was 92° F. or over, and the relative humidity was 40 per cent or more, the thermal stress to the patient was found to be excessive. However, in general, the relative humidity of the room did not appear to be a critical factor.

By using control solutions the method was found to be readily reproducible with a technical error no greater than that for serum electrolytes. When the same subject was tested more than once the sweat electrolytes were noted to be relatively constant.

It is recommended that the patient be under constant observation during the test period. Although no ill effects from the test were noted in this group of patients, deaths have been reported. The relative contraindications include: fever, dehydration, vomiting, diarrhea, diuresis, peripheral vascular insufficiency, steroid therapy, mental disorders, and marked cardiac or pulmonary insufficiency.

TABLE V

NORMAL VALUES FOR SWEAT ELECTROLYTES IN MILLIEQUIVALENTS PER LITER OF SWEAT

AUTHOR	NO. TESTS	CHLORIDE		SODIUM		METHOD
		RANGE	MEAN	RANGE	MEAN	
Peterson	55	4-66	28	8-72	32	Cellulose sponge
di Sant'Agnese[8]	226	4-60	27	10-80	52	Cotton gauze
Kaiser et al.[9]	40	3-59	22	3-58	21	Cotton gauze
Barbero et al.[6]	31	8-93	36	1-88	26	Cotton gauze

Fifty-five subjects who were either normal individuals not related to a patient with cystic fibrosis or patients with an illness dissimilar to cystic fibrosis were used as controls. None of these patients had evidence of respiratory, pancreatic or allergic diseases. Likewise, there was no evidence of adrenal disease or peptic ulceration in the control group. The average age was 32.5 years with a range from 9 months to 87 years. Normal for the cellulose sponge method for chloride ranges from 4 to 66 mEq. per liter with a mean of 28 mEq. per liter. Sodium ranges from 8 to 72 mEq. per liter with a mean of 32 mEq. per liter. These values are compared with those of several authors in Table V.

SUMMARY

A new method for the collection of sweat in pure form for electrolyte concentration determination has been described together with supporting data. This method is simpler and quicker than the commonly employed method using cotton gauze.

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FREQUENCY OF RECOVERY OF BETA HEMOLYTIC STREPTOCOCCI AS RELATED TO NUMBER OF THROAT SWABS

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THE frequency of recovery of beta hemolytic streptococci from throat cultures has been shown[1] to depend on the number of swabs taken simultaneously. In a prior study, during which duplicate swabs had been collected, approximately one-third of the group A organisms isolated would have been missed had a single swab been used. Each of the two swabs had been planted on a different media, one on Difco blood agar base enriched with 4 per cent defibrinated sterile sheep cells, and the other, on Difco neopeptone heart infusion agar, also enriched with 4 per cent defibrinated sheep cells.

In a subsequent investigation,[2] we observed that approximately 25 per cent of group A beta streptococci failed to appear on one or other of the 2 plates used. We were unable to discern any specific type or morphologic predilection for one media over the other.

For epidemiologic purposes, a further check of accuracy of recovery rates is essential. The present study was carried out to determine whether or not more streptococcal isolations would result when the number of simultaneous throat swabs is increased.

METHODS

Throat cultures were taken from 274 subjects in Miami, Florida, during May, 1956. At the beginning of the investigation, 4 sterile cotton applicators were swabbed simultaneously over the tonsillar fauces and posterior pharynx of each individual. However, because of the bulk of these applicators, 2 consecutive swabbings with 2 applicators for each sampling were performed in the later portions of the study. Of the first pair of swabs, one was streaked on a Difco blood agar base plate enriched with 4 per cent defibrinated sheep blood cells (BAB), and the other swab on a Difco neopeptone heart infusion agar plate (NEO) similarly enriched with sheep blood cells. The second pair of applicators was treated in the same way, one being streaked on blood agar base and the other on neopeptone heart infusion agar. All streaking was done within 2 hours of collection of samples. Plates were incubated at 37° C. for 24 hours and examined for the presence of beta hemolytic streptococci, as previously described.[1-4]

All beta streptococci recovered were grouped at the Florida State Board of Health with antiserum kindly provided by Dr. Elaine Updyke, Communicable Disease Center Laboratories, United States Public Health Service, Chamblee, Ga.

From the Department of Medical Research, National Children's Cardiac Hospital, Miami, Fla.

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TABLE I. DISTRIBUTION OF 58 CULTURES POSITIVE FOR BETA HEMOLYTIC STREPTOCOCCI

AGE	TOTAL CULTURES STUDIED	TOTAL NEG. CULTURES	POSITIVE FOR BETA						
			ON I PLATE ONLY	ON N PLATE ONLY	ON C PLATE ONLY	ON D PLATE ONLY	ON I AND N PLATES	ON I AND C PLATES	ON I AND D PLATES
Adults				A(2) G(2)	G(15)	C(2)			
	76	71		2	1	1			
Children 6 to 9 years old			F(4)	NG(4) NG(1) A(4)	A(50)	A(1) A33(1,000) C(5) F(200) G(3) NG(1)	A(1/100) A(100/3)	A(1/10)	I=A(1,000) D=G(1)
	98	66	1	3	1	6	2	1	1
Age group 13 to 15 years old			G(10) NG(10)	A(2) B(50) G(1)	A(1)	A(100) A(4) NG(5)	G(1/1)	A(15/1) C(2/25)	
	100	79	2	3	1	3	1	2	
Total	274	216	3	8	3	10	3	3	1

Key to abbreviations: I = first BAB plate; N = first NEO plate; C = second BAB plate; D = second NEO plate. Parentheses = number of colonies on initial plate; (x/y) = x, number of colonies on first plate; y, number of colonies on second plate. A, B, C, F, G, NG = Beta hemolytic streptococci groups A, B, C, F, G, or nongroupable.

RESULTS

Of the 274 throats cultured, 58 (21 per cent) yielded beta hemolytic streptococci, while 217 were negative for these organisms in all 4 plates. Only 12 of the 58 positives were recovered on all 4 plates, while 24 (41 per cent of the 58) were isolated from a single plate only (Table I). Our procedure of using a double swab routinely would have failed to recover 18 of the 58 strains of streptococci (31 per cent).

Group A strains were recovered 31 times; on 3 occasions other groups were isolated simultaneously. Had the double swab technique been employed, 9 such strains would have been missed (29 per cent).

The numbers of colonies observed on each plate also are recorded in Table I. Of the positive cultures for group A organisms, these numbers varied from 1 colony (e.g., on the C plate of the age group 13 to 15 years old) up to 1,000 colonies (e.g., the A_{33} isolate on plate D in the 6- to 9-year-old children).

DISCUSSION

The use of 4 throat swabs instead of 2 increased the frequency of isolation of beta hemolytic streptococci by 30 per cent, both for all groups of streptococci recovered, and also for group A organisms alone. The better yield was anticipated, since we previously had observed that the use of 2 swabs resulted in the recognition of positive cultures 30 per cent more frequently than when a single swab was used.[2, 4]

In streptococcal epidemiologic studies, taking 4 swabs from each throat creates a great deal of work, which may not be justified. However, the investigator should take cognizance of the inherent errors in throat culturing

AS ISOLATED FROM 4 SIMULTANEOUSLY TAKEN THROAT SWABBINGS

HEMOLYTIC STREPTOCOCCI						
ON N AND C PLATES	ON C AND D PLATES	ON I, N, AND C PLATES	ON I, C, AND D PLATES	ON I, N, AND C PLATES	ON N, C, AND D PLATES	ON I, N, C, AND D PLATES
					A(10/1/2)	
					1	
A(1,000/100)	A(2/4) A(3/1)	A(2/1/1) F(25/100/5)	A(50/3/50) I = F(1) C = F(1) D = A(3)	G(2/1/3) G(1/10/2)		A(1,000/50/100/50) A(50/35/1,000/2) A(1/15/1,000/1,000) G(1,000/100/1,000/1,000) A(1/10/25/8) A(25/30/6/1) *
1	2	2	2	2		† 8
	C=NG(2) D=B(1) G(25/3) A(1/4)		NG(3/100/10)		A(3/3/1)	A33(3/1/10/50) G(1/1,000/40/15) G(1,000/1,000/1,000/1,000) ‡
	3		1		1	4
1	5	2	3	2	2	12

*One culture I = A(5); N = G(10); C = G(10); D = A(10).
†One culture, I = C(1,000); N = C(1,000); C = C(100); D_x = C(1,000); D_t = G(1,000).
‡One culture, I = G(4); N = NG(100); C = G(1,000); D = G(4).

techniques, and recognize that the larger the number of swabs taken from an individual subject, the greater is the likelihood of isolation of beta streptococci. The magnitude of the increased recovery rate is considerable.

This increase in frequency of obtaining positive cultures for beta streptococci is not necessarily a function of the number of colonies observed, nor of the media employed, nor of the group of streptococci involved.

There may be several reasons for the higher rates of streptococcal isolation with increasing numbers of swabs: (1) simple chance, which increases the number of opportunities to pick up unevenly scattered colonies, appears to be the most important consideration (e.g., a swab may miss an area of the pharynx or tonsil where large masses of streptococci may be present, while covering many areas free of these organisms); (2) a particular colony may grow better on one type of media than on another; (3) the streaking of the swab on the plate may fail to plant colonies of streptococci already on the cotton; (4) death of organisms may occur between time of collection and of streaking; (5) the observer may fail to recognize beta streptococci because of atypical colonial morphology or overgrowth by other colonies.

Variations in number of colonies of streptococci may be due almost entirely to chance contact of swab with contaminated pharyngeal and tonsillar areas, and swab with plate at time of streaking.

The present observations emphasize the importance of appreciation of the magnitude of errors that may occur during routine throat culture studies. They also point to the need for a more accurate technique for culturing beta hemolytic streptococci, a problem which we are currently investigating.

SUMMARY

Of 274 throat cultures taken with quadruplicate swabbing, 58 were positive for beta hemolytic streptococci.

Considering all groups of beta streptococci isolated, 31 per cent of cultures would have been lost if only duplicate swabs were taken; of group A organisms, 29 per cent would have been lost.

The differences observed cannot be attributed to the numbers of colonies recovered. Chance seems to explain the increased frequency of isolation with an increased number of swabs.

These data provide warning to the epidemiologist of the danger of relying on the usual throat culture techniques to give more than an indication of streptococcal recovery rates.

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USE OF BROMELIN IN BLOOD TYPING

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THERE is ample evidence that exposing red blood cells to the action of certain proteolytic enzymes alters their reaction patterns so that agglutination with incomplete antibodies is observed without having to resort to the Coombs' test.[1-3] The enzymes in most common use are trypsin, papain, and ficin, with papain being the only one suitable for direct use in capillary tests.[4] Since satisfactory results with papain can be obtained only if an activated solution is used, papain has the disadvantage for field studies in that the activated solution must be prepared in advance and kept frozen until needed.[5] The following study represents results obtained using bromelin, a proteolytic enzyme obtained from pineapple pulp.

A brief mention of the effect produced by fresh pineapple juice on red cell agglutinogens has been previously published by Wheeler and associates.[6]

The present objective was to determine if bromelin could be standardized for use in both capillary and tube tests without having to resort to an activation process. The capillary method, as compared to tube techniques, offers distinct advantages in saving of time and antisera. The latter point is important in that small amounts of rare and expensive typing sera can be used for typing large population samples.

MATERIALS AND METHODS

1. The bromelin used in this study was kindly provided by the Wallerstein Laboratories, New York, N. Y.

2. The antisera used were the following.

   a. Anti-D, 5 samples of incomplete sera.
   b. Anti-C, 4 samples of incomplete sera.
   c. Anti-c, 5 samples of incomplete sera.
   d. Anti-E, 4 samples of incomplete sera.
   e. Anti-E, 2 samples of incomplete sera.
   f. Anti-K, 3 samples of incomplete sera.
   g. Anti-Fy^a, 4 samples of incomplete sera.
   h. Anti-Le^a, 1 sample of complete serum.
   i. Anti-P, 2 samples of human complete sera.
   j. Anti-S, 1 sample of complete serum.
   k. Anti-M, 3 samples of rabbit immune sera and one sample of human serum.
   l. Anti-N, 3 samples of rabbit immune sera.
   m. Anti-Jk^a, 1 sample of incomplete serum.
   n. Anti-A, 3 samples of complete sera.
   o. Anti-B, 3 samples of complete sera.

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p. Anti-H, 1 lot of Ulex europeus from which repeated fresh extracts were prepared.

q. Coombs' sera, 1 sample of rabbit immune human serum and 3 samples of rabbit immune human gamma globulin.

The anti-Le^a, anti-S, and one sample of anti-Fy^b were kindly provided by Dr. J. L. Griffits of Dade Reagents, Inc. Two samples of anti-K and anti-Fy^a were provided by Dr. Bruce Chown. All other typing sera were purchased from commercial suppliers.

3. For each antiserum, at least 15 positive and 15 negative red cell blood samples were available. The laboratory panel was checked against Panocell and Hemantigen obtained from Kniekerbocker Biologicals, Inc., New York, N. Y.

4. It has been found that a special slant box for reading capillary tests is not essential. An ordinary illuminated viewing box can be used by propping at an angle and adding a layer of Plasticine to the lower edge.

Methods.—

1. Capillary test for Kell and Rh typing: The method of loading of the capillaries is identical with that outlined by Lewis and associates.[4] One half inch of antiserum is drawn into a capillary followed by twice that amount of 0.1 per cent saline solution of bromelin. Last, not more than ¼ in. of a 50 per cent suspension of red cells is drawn up. The tube is quickly inverted and stuck in Plasticine at an angle.

2. Test tube method for Rh typing: One volume of 0.1 per cent bromelin is added to 1 volume of a 2 per cent saline suspension of red blood cells. The tubes are incubated for 15 minutes as 37° C. The bromelin is removed by filling the tubes with saline followed by centrifuging and discarding the saline. One drop of fresh saline is then added to reconstitute the 2 per cent suspension by a drop of antiserum. Incubation is not usually necessary.

3. Controls: In addition to checking the reactions of known positive and negative red blood cells with each antiserum, sensitized cells should also be tested with AB serum to detect possible overtreatment with bromelin. In practice, bromelin used in the recommended dilution shows little tendency to oversensitize. Incubation periods up to 2 hours failed to produce agglutination in AB serum. The latitude is certainly greater than that encountered with trypsin or ficin.

RESULTS AND DISCUSSION

The ABH, Le^a, S, Rh, Kell, and P antigens exhibit greater reactivity when red blood cells are treated with bromelin. Incomplete Rh and Kell antisera produce spontaneous agglutination of treated red blood cells suspended in saline. Kell and Rh tests performed in capillary tubes will show agglutination of positive cells within 10 minutes. False positive reactions do not appear even if the capillary tubes are left overnight.

Bromelin treatment results in a three- to fourfold increase in A and B activity as measured by the strength of agglutination of treated and untreated red cells added to sucessively doubled dilutions of anti-A and anti-B. Of interest is the four- to fivefold increase of H activity for enzyme treated cells of all ABO types as measured by the titers obtained in Ulex extract (anti-H). Although all types produce an increase in titer when treated with bromelin, the quantitative differences in amount of H antigen are not obscured. Treated type O red cells still give the highest agglutination titer followed by types A_2, A_2B, B, A_1, and A_1B treated red cells in that order.

The quantitative difference in strength of agglutination is also maintained between bromelin-treated type A_1 and A_2 red cells when both are titrated against the same anti-A.

The enhancing action on Le^a, S, and P reactions was, for the few serum samples tested, so slight as to be of little value.

A single sample of incomplete anti-Jk^a agglutinated Jk^a/Jk^a bromelin-treated cells suspended in saline but did not agglutinate similarly treated heterozygous cells. The indirect Coombs' test was however much stronger with treated heterozygous cells than with nontreated heterozygous cells.[7]

The MN and Duffy antigens are denatured by bromelin in the same manner that they are denatured by trypsin, papain, and ficin. The reactions of rabbit immune anti-M and N with treated cells are both weakened and nonspecific. Negative reactions occurred with human anti-M and treated type M cells.

One special word of caution must be mentioned with reference to the use of enzyme-treated cells in blood typing. All typing sera should preferably contain only one antibody. If a mixture of antibodies is present, as is usually the case, the sera must be completely absorbed of all but the one desired and checked against a panel of enzyme-treated cells. As the reactivity of the A, B, and Rh antigens is increased, any residual antibodies to these factors can cause difficulty even though no reaction can be detected with untreated cells.

SUMMARY

A satisfactory method for detection of the Kell and Rh antigens using the capillary test has been described. Enzyme treatment of red blood cells is produced by exposure to 0.1 per cent bromelin in saline. The effect of bromelin treatment on other blood group systems is described.

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A MICROCOLORIMETRIC METHOD FOR THE DETERMINATION OF SORBITOL, MANNITOL, AND GLYCEROL IN BIOLOGIC FLUIDS

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Baltimore, Md.

THE metabolism of sorbitol and mannitol in mammalian tissues is, as yet, incompletely understood. We have found recently that both these sugar alcohols and glycerol can replace glucose in the medium used for the growth of a strain of mammalian tumor cells (the MB III strain of mouse lymphoblasts[1]) maintained in vitro in roller tube cultures. The amount of cellular material normally present in these cultures is of the order of 0.2 mg. (one million cells), and in order to measure the utilization of sorbitol and mannitol a method was required for determination of quantities in the range 10 to 20 μg.

Methods previously reported have been based upon the procedure of Rappaport and associates.[2] In this method the sugar alcohol is oxidized with periodic acid and the excess periodate is back titrated with arsenite or thiosulfate. From 3 to 5 mg. of material is required. Another method[3] utilizes ceric sulfate oxidation of the alcohol followed by measurement of excess ceric ion by titration. The method is less specific than the periodate procedure and again requires relatively large amounts of material.

The oxidation of sugar alcohols by periodate follows the equation:

$$(CH_2OH)_2 \cdot (CHOH)_4 + 5HIO_4 \rightarrow 2HCHO + 4HCOOH + 5HIO_3 + H_2O$$

where primary hydroxyl groups each yield 1 mole of formaldehyde, and the secondary hydroxyl groups yield 1 mole of formic acid. This has been used as the basis of a method for the determination of glycerol (Lambert and Neish[4]), where the formaldehyde formed is determined by using the chromotropic acid colorimetric reagent.

In the method described in this paper the deproteinized solution containing sorbitol, mannitol, or glycerol is treated with periodic acid in acid solution for a limited time to prevent oxidation of glucose. The excess periodate is removed by addition of sodium arsenite solution and the formaldehyde is determined colorimetrically by using a modification of the phenylhydrazine-ferricyanide procedure of Kersey.[5] The method is suitable for the determination of 5 to 50 μg of sorbitol, mannitol, or glycerol in 0.1 ml. of culture fluid without interference from other components of the medium.

MATERIALS AND METHODS

All materials were of analytical grade where available. Sorbitol and mannitol were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The samples were free from glucose and other reducing sugars and were used without further purification.

From the Finney-Howell Cancer Research Laboratory, Department of Surgery, Johns Hopkins Medical School, Baltimore, Md.
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Reagents.—

1. Sulfuric acid, 1 N.
2. Periodic acid, 0.2 M.
3. Sodium arsenite solution, 1 M, prepared by dissolving 2.5 Gm. sodium hydroxide pellets and 5 Gm. arsenious oxide powder in 50 ml. water.
4. Phenylhydrazine solution (0.4 per cent), prepared by dissolving 400 mg. phenylhydrazine hydrochloride in 100 ml. 0.4 N HCl.
5. Potassium ferricyanide solution (5 per cent).
6. Hydrochloric acid, 4 N.
7. Barium hydroxide solution (0.3 N).
8. Zinc sulfate solution (5 per cent) containing 0.004 per cent phenol red.
9. Magnesium sulfate solution (4 per cent).

Reagents numbers 2, 3, 4, and 5, stored at 4° C., are stable for up to 3 months.

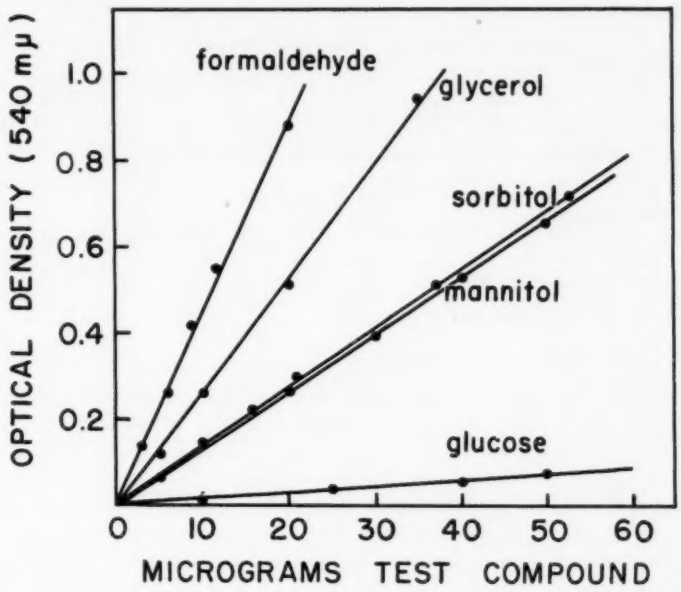

Fig. 1.—Calibration curves for colorimetric determination of sugar alcohols.

Procedure.—A sample of culture fluid (0.1 ml.) containing 5 to 50 μg of sorbitol, mannitol, or glycerol is diluted to 1 ml. with water and deproteinized by the addition of 0.2 ml. of barium hydroxide solution followed by 0.18 ml. of the zinc sulfate reagent. At this stage a slight excess of barium hydroxide should be present in order to remove zinc completely from the solution. The solution should therefore be a decided pink color and more barium hydroxide is added if required. The excess barium is removed by addition of 0.05 ml. magnesium sulfate solution. To a 1 ml. portion of the supernatant obtained after centrifugation is added 0.4 ml. 1 N sulfuric acid and 0.1 ml. periodic acid solution. Oxidation is allowed to proceed for exactly 10 minutes and the reaction is then arrested and excess periodate decomposed by addition of 0.2 ml. sodium arsenite solution. After one or two minutes the color of the liberated iodine appears and slowly fades. The colored phenylhydrazine-ferricyanide complex is formed by addition of 1 ml. phenylhydrazine reagent and 0.1 ml. potassium ferricyanide solution, and the mixture is allowed to stand for

exactly 10 minutes to allow full color development. Hydrochloric acid (4 N, 2.3 ml.) is then added to intensify and stabilize the color, and the optical density of the magenta colored solution is measured after a further 5 or 10 minutes at a wave length of 540 mμ, using the 1 cm. cells of the Beckmann model DU spectrophotometer. The sorbitol, glycerol, or mannitol content of the solution is calculated by comparison with standard curves obtained by treating samples of known content by the procedure described above (Fig. 1). The whole procedure subsequent to the deproteinization is conveniently carried out in a 13 by 100 mm. Pyrex test tube of 5 ml. capacity.

TABLE I. COLORIMETRIC DETERMINATION OF SUGAR ALCOHOLS

A. Rate of Formation of Phenylhydrazine-Ferricyanide Complex

Time before adding HCl (min.)	1	5	10	15	45
Optical density (540 mμ)*	0.133	0.390	0.430	0.435	0.440

B. Stability of Color Formed From Oxidation Products of Sorbitol

Time after adding HCl (min.)	1	2	3	5	10	17	30	40	55
Optical density (540 mμ)†	0.205	0.220	0.235	0.237	0.233	0.227	0.209	0.198	0.191

C. Determination of Sorbitol in Tissue Culture Medium in Presence of Glucose

Micrograms sorbitol put in‡	0	4.5	9.0	13.5	18	18	27	36	45	45
Micrograms sorbitol found	0	3.9	9.2	15.3	18	17.8	27.4	35.2	44.7	45.0

*Reaction mixture contained 7.6 μg of formaldehyde.
†Reaction mixture contained originally 15 μg of sorbitol.
‡In addition, 40 μg of glucose was also present.
Other additives were as described in the text.

DISCUSSION

It is essential during the deproteinizing procedure to remove zinc and barium ions since these both form a precipitate with the ferricyanide reagent and thus interfere with the final color development. A slight excess of barium hydroxide is therefore added to remove all the zinc. The excess barium is then removed by addition of magnesium sulfate, and since magnesium does not interfere with the ferricyanide reagent, an excess may be present. It has been our experience with different samples of serum and other components of tissue culture media, that the relative ratios of barium hydroxide to zinc sulfate reagent required for deproteinizing may change, mainly because of variations in the bicarbonate content of the test solution. Solutions of high bicarbonate content require more barium hydroxide. The procedure outlined above, by allowing the addition of a moderate excess of barium in all cases, overcomes the disadvantage of having to determine an exact equivalence between the deproteinizing reagents for each test solution.

The conditions for maximum and stable color development with formaldehyde are quite critical. If hydrochloric acid is added too soon after addition of the phenylhydrazine-ferricyanide reagents, maximum color development is inhibited (Table I, *A*). The optimum time was found to be about 10 minutes standing with the reagents before addition of acid. Heating the solution to accelerate phenylhydrazine formation gave a less intense final color. After

addition of hydrochloric acid, about 3 to 5 minutes are required for the intensity to reach a maximum. The color is stable for a further 5 minutes and then slowly fades (Table I, *B*). It is also important that an excess of arsenite is present and that reduction of excess periodate is complete. Oxidation of phenylhydrazine by periodate will otherwise interfere with subsequent color development.

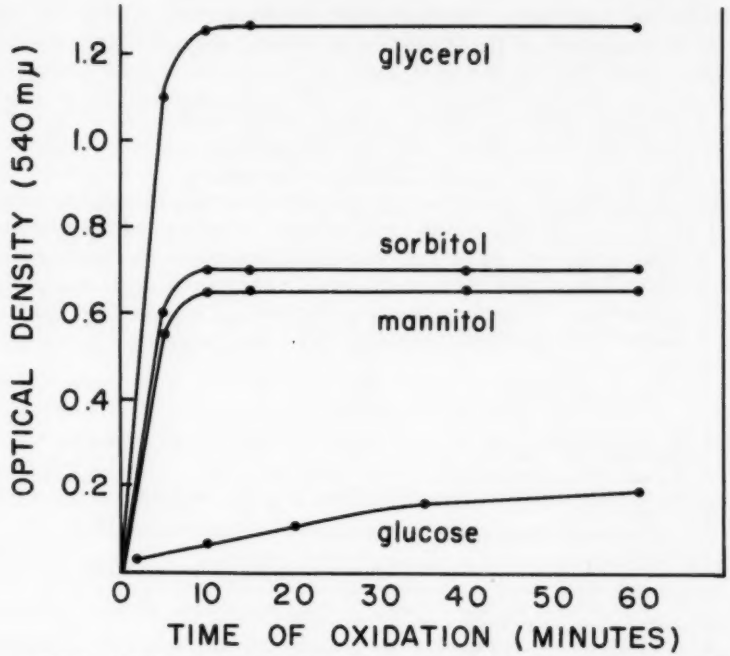

Fig. 2.—Oxidation rates of sugar alcohols compared with glucose. Fifty micrograms of each compound was reacted with periodic acid at room temperature for different time intervals. Reaction was arrested by addition of excess sodium arsenite solution and the formaldehyde formed was determined colorimetrically as described in the text.

The relative rates of oxidation of sorbitol, mannitol, glycerol, and glucose are shown in Fig. 2. From this it can be seen that oxidation of the polyhydric alcohols is complete within 10 minutes, under the conditions used for the determination, whereas oxidation of glucose proceeds at a much slower rate. It is thus possible to determine these substances in the presence of an equal amount of glucose. If an appreciably greater quantity of glucose is present, it may be determined separately by the Nelson-Somogyi colorimetric copper reducing method,[6] a procedure which gives no color with sugar alcohols. A correction may then be applied to the oxidation procedure by using the data in Fig. 1. In Table I, *C*, for example, the results of a number of determinations of sorbitol carried out in the presence of glucose are recorded. Varying amounts of sorbitol (5 to 45 μg) were added to 0.2 ml. portions of

a tissue culture medium. The medium consisted of 10 per cent human placental cord serum, supplemented with 50 per cent of Eagle's synthetic medium (a mixture of 12 amino acids and 6 vitamins)[7] and 40 per cent of a balanced salt solution. The glucose content of each 0.2 ml. sample was 40 μg as determined by the copper reducing method.

The method gives good results in the range 5 to 40 μg glycerol and 5 to 50 μg sorbitol and mannitol. Above these limits the calibration curves shown in Fig. 1 become nonlinear. We have used the procedure successfully for the routine determination of the utilization of sorbitol, mannitol, and glycerol by cultures of the MB III strain of mouse lymphoblasts during prolonged periods of in vitro growth. The results of these experiments will be reported elsewhere.

SUMMARY

A rapid microcolorimetric method for the determination of sorbitol, mannitol, and glycerol in biologic fluids is described. The procedure employs periodate oxidation followed by a colorimetric determination of the formaldehyde formed, and is suitable for 5 to 50 μg of material. Glucose and other blood serum components do not interfere, and the method may be used for the dual determination of glucose and the polyhydric alcohols present in a single sample.

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A SIMPLE CROSS TRANSFUSION AND PERFUSION PUMP FOR SMALL ANIMALS

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DURING the course of some studies on leukopenia in rabbits, it became desirable to cross transfuse several pairs of animals. The nature and short duration of the experiments required a quickly made inexpensive perfusion pump, preferably made from materials at hand. To satisfy these conditions, the pump described below was constructed. The device is operated by changes in air pressure and, except for the flutter valves, has no moving parts in contact with the circulating fluid.

The body of the pump consists of a Pyrex glass cylinder with the attachments, openings, and dimensions given in Fig. 1. The activating force is the change of pressure induced within the pump by advancing or withdrawing the plunger of the 10 or 20 ml. syringe. Air seal is obtained by coating the plunger of the syringe with sterile mineral oil. Dental dam flutter valves described below are tied to the ends of the inlet and outlet tubes as indicated in the figure. The outlet valve of the pump is housed in a small glass cylinder fitted to the stem of the pump body by a rubber stopper. The actual volume of blood or fluid passed through the pump on each pulsation is read directly from a calibrated scale affixed to the pump body.

The flutter valve is a readily constructed directional flow valve and may be made to almost any size requirement. It consists of two flat strips of dam which are sealed along the edges and, when not dilated by inside pressure, lying in close approximation to each other. Proper operation, however, requires that such valves be free of built-in deformities, such as wrinkling, kinking, or local stretching. To meet these requirements in the making of small, fragile valves, a technique using filing cards as splints during the processing was developed. The successive steps in the making of the valve follow.

METHODS

A film of rubber cement is placed on one surface of each of two 3 by 5 inch filing cards and allowed to become tacky. The cemented surface of each card is then placed in contact with a sheet of powder-free (washed and dried) dental rubber dam lying on a flat surface. Uniform contact and adhesion are obtained by rolling with a glass or other cylinder. The card-supported sheets are now perfectly flat and can be manipulated without

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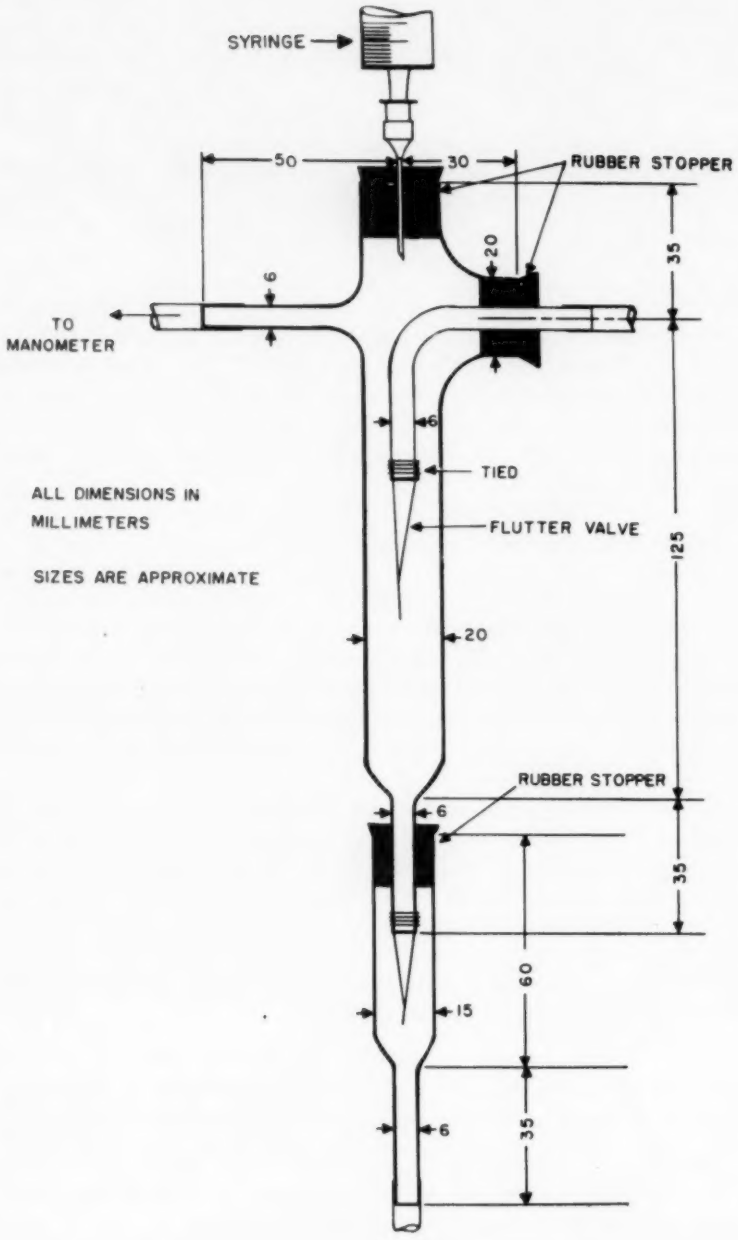
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SIZES ARE APPROXIMATE
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20
RUBBER STOPPER
6
35
60
15
6
35

Fig. 1.

the introduction of warping or stretching. Next, strips from a third filing card are cut of a width equal to the diameter of the tubing to be inserted into the valves. The strips are powdered on both sides with talcum powder and the excess removed. The dam surfaces of the card-backed sheets are next coated with rubber cement. This is applied evenly and thinly with the finger tip over the entire surface. Excessive amounts of cement immediately blister the dam and render it useless. As indicated in Fig. 2, the powdered card strips B are then placed on the cemented face of one sheet A, with a free space of at least 1 cm. The second sheet (C) is placed so that the cemented faces are in contact.

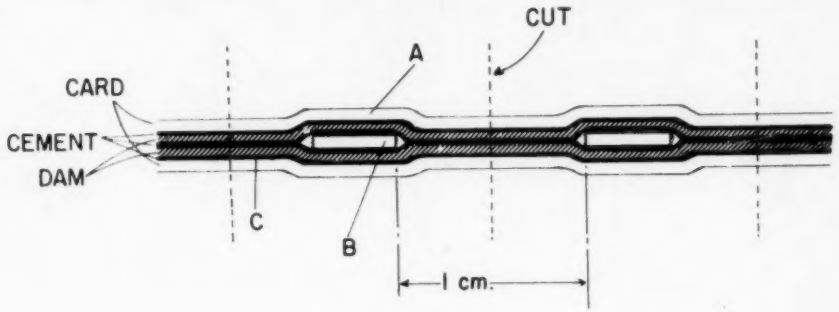

Fig. 2.

Close adherence is sought, particular attention being paid to the sides of the enclosed strips. When full adhesion is obtained, the card-dam sandwich is cut parallel to and midway between the powder strips, giving a number of card-backed flutter valves. The backing is peeled away, the powder strip removed and the valves powdered and stretched to remove the kinking formed during the peeling. The properly made valve now lies perfectly flat and following washing to remove the talcum powder is ready for mounting.

If necessary, the rubber flutter valve may be replaced by a valve of polyethylene made from thin sheeting or obtained as thin-walled tubing. The exacting temperature requirements make construction from the sheeting difficult while the commercial tubing fails to lie flat at the angles so that complete prevention of backflow may not be obtained.

DISCUSSION

The pump as described above has been successfully used in cross transfusions in heparinized rabbits. The inlet tubing is connected with polyvinyl clinical transfusion tubing and the latter is passed through the external jugular deep into the superior caval vein in the donor rabbit. Deep penetration of the venous system is essential for free filling of the pump on aspiration. The outlet is connected to polyvinyl transfusion tubing which in turn is tied into the external jugular of the recipient. A pair of pumps has been used in the cross transfusion of rabbits for as long as an hour during the course of which the circulating blood was exchanged several times. At the end of this procedure, small deposits of fibrin were found on the contacting surfaces of the valves but no other difficulties were encountered.

SUMMARY

A simple, easily made glass perfusion pump is described. The pump is activated by changes in air pressure within the pump body induced by a clinical

syringe. Flow direction is controlled by flutter valves made of dental dam. The construction of the flutter valves is given in detail since the method eliminates many of the difficulties usually involved and is applicable to the construction of fine flutter valves for a variety of purposes.

I wish to acknowledge the aid of Dr. J. R. Barberio who suggested several features in the design of the pump.

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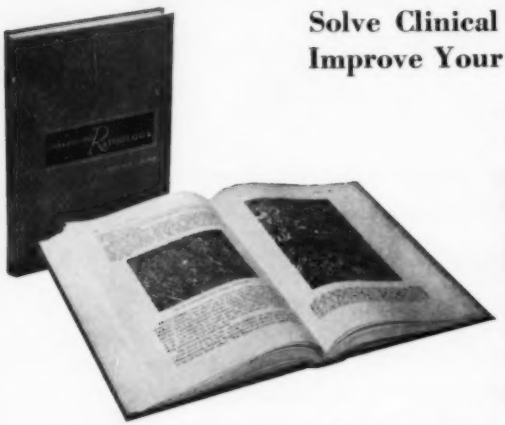

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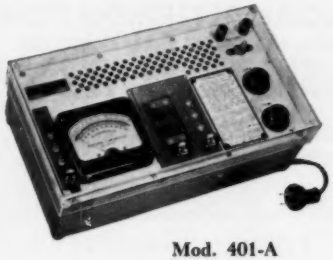

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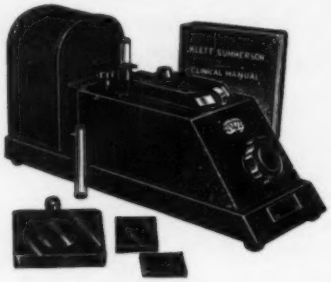

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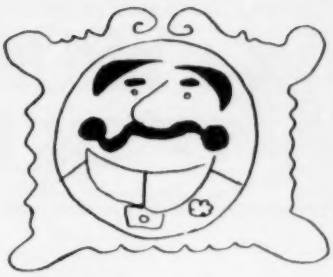

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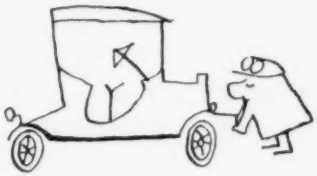

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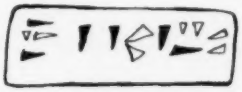

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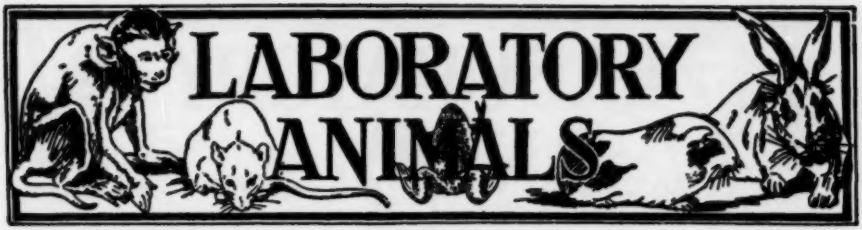

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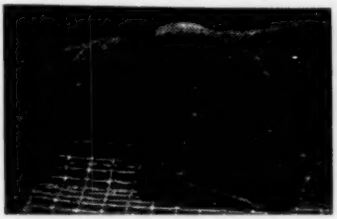

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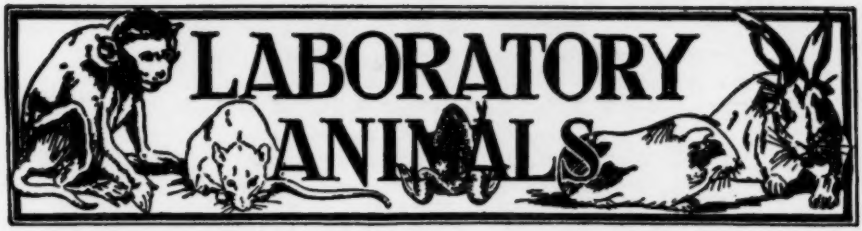

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